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This is EXHIBIT FIERS-39

to
 it of Walter C. Fiers
 in before me
 ay of November, 2001

or Oath or Notary Public

SUGANO EXHIBIT 1004
 FIERS V. SUGANO
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MAILING LIST FOR "Interferon" Manuscripts

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This is EXHIBIT FIER-39

to
 the Affidavit of Walter C. Fiers
 sworn before me
 this 13th day of November, 2001

Commissioner for Oath or Notary Public

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5. Wood, J. A. *The Solar System* (Prentice-Hall, Englewood Cliffs, 1979).
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13. Hayashi, C. *Proc. Symp. Aeronautical Inst. Univ. Tokyo (in Japanese)* **13** (1972).
14. Goldreich, P. & Ward, W. R. *Astrophys. J.* **183**, 1051 (1973).

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Synthesis in *E. coli* of a polypeptide with human leukocyte interferon activity

Shigekazu Nagata, Hideharu Taira, Alan Hall, Lorraine Johnsrud, Michel Streuli, Josef Ecsödi, Werner Boll, Kari Cantell* & Charles Weissmann

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* Central Public Health Laboratory, SF 00280 Helsinki, Finland

Double-stranded cDNA prepared from the 12S fraction of poly(A) RNA from interferon (IF)-producing human leukocytes was cloned in *Escherichia coli* using the pBR322 vector. One of the resulting clones had a 910-base pair insert which could hybridise to IF mRNA and was responsible for the production of a polypeptide with biological IF activity. Up to 10,000 units IF activity per g of cells was obtained from some clones.

CELLS of almost all vertebrates, when exposed to certain viruses or inducers, produce one or more (glyco)proteins, known as interferons^{1,2}. Interferons (IFs) are characterised biologically by their ability to induce in target cells a virus-resistant state which is associated with the *de novo* synthesis of several proteins, in particular a protein kinase³, an oligoadenylate synthetase^{4,5} and a phosphodiesterase⁶. In addition, IFs have a regulatory effect on the immune response⁷ and their enhancement of

killer lymphocyte activity⁸ may be the basis of their inhibitory effect on tumour growth⁹.

Two major classes of acid-stable (type I) IFs have been recognised in man—leukocyte interferon (Le-IF), released by stimulated leukocytes, and fibroblast interferon (F-IF), produced by stimulated fibroblasts. Le-IF and F-IF differ not only immunologically but also in their target cell specificity: whereas both IFs induce a virus-resistant state in human cells, Le-IF is also very active on bovine, porcine and feline cells, whereas F-IF is not². The two IFs are encoded by separate mRNAs¹⁰.

Human Le-IF has been purified more than 80,000-fold, to a specific activity of 4×10^4 units per mg (ref. 11) or 2.5×10^4 units per mg (ref. 12). Two components have been characterised by polyacrylamide gel electrophoresis, with apparent molecular weights (MWs) of 21–22,000 and 15–17,000, respectively^{13,14}; they are believed to differ in their degree of glycosylation¹⁴. Enzymatic¹⁵ or chemical¹⁶ removal of most or all of the carbohydrate moiety seems to have little effect on the biological activity of IF.

We sought to clone human Le-IF cDNA in order to construct bacterial strains producing polypeptide(s) with human IF activity, and to generate the tools required for the analysis of Le-IF gene structure and function. The particular difficulties of this undertaking were the lack of a purified Le-IF mRNA and our ignorance of the structure of Le-IF, which precluded the preparation of pure or highly enriched IF cDNA, or of a probe for the identification of the desired clones.

We describe here the isolation of a hybrid plasmid containing a 872-base pair Le-IF cDNA, which elicits the formation in *Escherichia coli*, of a polypeptide with the immunological and biological properties of human Le-IF.

Isolation of hybrid plasmids containing IF cDNA sequences

Hybrid DNA, consisting of leukocyte cDNA sequences joined to pBR322 at the *Pst*I site by means of dG:dC sequences, was prepared by conventional means, using as starting material a 12S fraction of poly(A) RNA from IF-producing leukocytes, purified about 10-fold for IF mRNA. cDNA cloned in this fashion is usually flanked

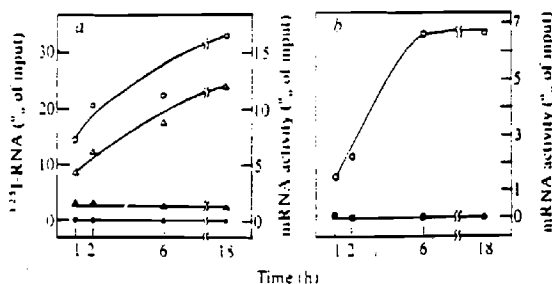


Fig. 1 Hybridisation of IF mRNA and ¹²⁵I-globin mRNA to filter-bound DNAs. DNA was linked to DPT paper as described elsewhere¹⁰. **a**, For each time point, one 0.25-cm² piece of DPT paper with 500 ng of HindIII-excised insert of rabbit β-globin cDNA plasmid and one piece with 700 ng of HindIII-digested pBR322 were hybridised as a sandwich with 10 μl of hybridisation medium²² containing 200 ng ¹²⁵I-labelled globin mRNA. **b**, As above except that DPT papers contained 250 ng of HindIII-excised insert of rabbit β-globin cDNA plasmid and 250 ng of *Pst*I-excised insert of Hif-2h, respectively, and hybridisation was with 5 μg of Le poly(A) RNA in 10 μl. In all cases hybridisation, washing and elution were as described in ref. 22. The RNA was recovered, its ¹²⁵I radioactivity determined, and injected into 40–50 oocytes. In experiment **b**, oocytes were incubated with 50 μCi ³H-histidine. Oocyte supernatants were assayed for IF activity by the cytopathic effect reduction assay (see Table 3 legend). Le poly(A) RNA (1 μg) injected directly into 20 oocytes gave 2,700 units IF. For the determination of ³H-labelled globin formation, oocytes were homogenised, centrifuged and an aliquot of the supernatant electrophoresed through a 20% polyacrylamide gel¹⁷. The globin band was cut out and the radioactivity determined in toluene-based scintillator solution. 100 ng globin mRNA injected directly gave 100,000 c.p.m. ³H-globin. **a**, ¹²⁵I-RNA hybridised to β-globin cDNA (○) or to pBR322 (●). ³H-labelled β-globin formed in oocytes after injection of RNA hybridised to β-globin cDNA or (△) pBR322 (▲). **b**, IF activity formed in oocytes after injection of RNA hybridised to Hif-2h fragment (○) or β-globin cDNA (●).

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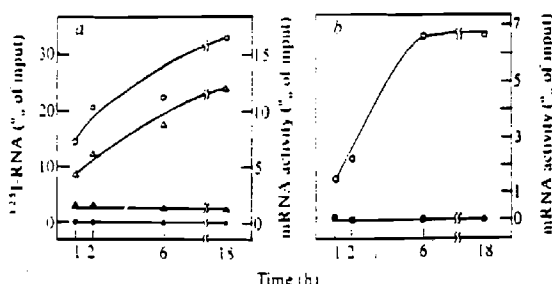


Fig. 1 Hybridisation of IF mRNA and ¹²⁵I-globin mRNA to filter-bound DNAs. DNA was linked to DPT paper as described elsewhere¹⁶. **a**, For each time point, one 0.25-cm² piece of DPT paper with 500 ng of *Hind*III-excised insert of rabbit β-globin cDNA plasmid and one piece with 700 ng of *Hind*III-digested pBR322 were hybridised as a sandwich with 10 μl of hybridisation medium²² containing 200 ng ¹²⁵I-labelled globin mRNA. **b**, As above except that DPT papers contained 250 ng of *Hind*III-excised insert of rabbit β-globin cDNA plasmid and 250 ng of *Pst*I-excised insert of *Hif*-2h, respectively, and hybridisation was with 5 μg of Le poly(A) RNA in 10 μl. In all cases hybridisation, washing and elution were as described in ref. 22. The RNA was recovered, its ¹²⁵I radioactivity determined, and injected into 40–50 oocytes. In experiment **b**, oocytes were incubated with 50 μCi ³H-histidine. Oocyte supernatants were assayed for IF activity by the cytopathic effect reduction assay (see Table 3 legend). Le poly(A) RNA (1 μg) injected directly into 20 oocytes gave 2,700 units IF. For the determination of ³H-labelled globin formation, oocytes were homogenised, centrifuged and an aliquot of the supernatant electrophoresed through a 20% polyacrylamide gel¹⁷. The globin band was cut out and the radioactivity determined in toluene-based scintillator solution. 100 ng globin mRNA injected directly gave 100,000 c.p.m. ³H-globin. **a**, ¹²⁵I-RNA hybridised to β-globin cDNA (○) or to pBR322 (●). ³H-labelled β-globin formed in oocytes after injection of RNA hybridised to β-globin cDNA or (Δ) pBR322 (▲). **b**, IF activity formed in oocytes after injection of RNA hybridised to *Hif*-2h fragment (○) or β-globin cDNA (●).

Table 1 mRNA hybridisation translation assay for the detection of IF cDNA in hybrid DNA from pools of transformed *E. coli*

DNA sample	Interferon activity
Expt 1: Pools of 512 clones	
I	<60 (<60); <u>110</u> (<20); <110 (<110); <110 (<110); <35 (<35)
δ	20 (<20)
N	35 (<20); <110 (<110); <u>200</u> (<110)
A	<60 (<60); <u>60</u> (<20); <110 (<110); <110 (<110)
8 other groups negative	
Expt 2: Pools of 64 clones from sample A	
A-I	<35 (<35); <35 (<35)
A-II	130 (<30); <45 (<45)
A-III	<u>125</u> (<35); <u>35</u> (<30); <u>35</u> (<30); <u>600</u> (<30); <20 (<20)
A-IV	<u>85</u> (<35); <25 (<25)
A-V to VIII	negative
Expt 3: Pools of 8 clones from sample A-III	
A-III-1	<20 (<20); <20 (60); <u>35</u> (<30)
A-III-2	<35 (<35); <30 (<30); <u>150</u> (<20); <u>600</u> (<35); <u>110</u> (60)
A-III-3	<25 (<25); <30 (<30)
A-III-4	<u>10</u> (<30); <20 (<20); <20 (60)
A-III-5 to 8	negative
Expt 4: Single clones from sample A-III-4	
A-III-4B	<35* (<35); <20 (60)
A-III-4C	35 (60); <u>60*</u> (<35); <u>111*</u> (<11); <11 (<11); <u>20</u> (<20)

Hybrid DNA containing leukocyte cDNA was prepared as follows. To obtain poly(A) RNA from IF-producing leukocytes, 10^{11} human leukocytes were primed with Le-IF and induced with Sendai virus as described elsewhere²¹. After 5 h at 37°C the cells were collected, suspended in 1 l PBS and added to 17 l 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2% SDS. The lysate was digested with Pronase (200 µg ml⁻¹) for 1 h at 20°C. 2 M Tris-HCl buffer (pH 9.5, 5% vol) was added and the solution extracted with 15 l phenol for 30 min. Chloroform (3 l) was added to aid phase separation, the aqueous phase adjusted to 0.3 M NaOAc buffer (pH 5.5) and the nucleic acid precipitated with ethanol. The precipitate (about 1 g) was dissolved in 900 ml TNE [Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA] containing 0.5% SDS, extracted three times with phenol and exhaustively with ether, and the poly(A) RNA recovered by three batch adsorptions to 3 × 5 g oligo (dT) cellulose (type 7, P-L Biochemicals) followed by elution with water. The yield was 1.6 mg; 1 µg gave rise to 300 units IF when injected into oocytes. For further purification, 860 µg RNA in 5 mM EDTA were passed through a Chelex-100 column, heated for 90 s at 100°C and centrifuged through a 5–23% sucrose gradient in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.2 M NaCl. The fractions containing the IF-mRNA activity, sedimenting around 12S, were pooled and the poly(A) RNA recovered by oligo(dT) cellulose chromatography. The yield was 40 µg; 1 µg gave rise to 3,600 units IF when injected into oocytes. pBR322-linked Le-cDNA was prepared essentially as described previously²². Sucrose-gradient purified poly(A) RNA from two preparations (48 µg) was used as template for reverse transcriptase to generate 10 µg cDNA 600–1,000 nucleotides long; this was converted into double-stranded DNA by DNA polymerase I, and treated with *S₁* endonuclease (yield, 8 µg preparation A). Of this DNA, 5 µg were centrifuged through a sucrose-density gradient, and material sedimenting faster than a 600-base pair ³²P-DNA marker was pooled and precipitated with ethanol (preparation B, 3 µg). cDNA was elongated with dCMP residues, annealed to dGMP-elongated, *Pst*I-cleaved pBR322 (ref. 17) and used to transform *E. coli* X1776 (ref. 23) (preparation A: 3.3×10^4 tetracycline-resistant transformants per µg DNA), or *E. coli* HB101 (preparation B: 4×10^4 transformants per µg DNA). Ten thousand colonies of transformed *E. coli* X1776 were inoculated individually into wells of microtitre plates and stored with 20% glycerol at –20°C. Five thousand colonies of transformed *E. coli* HB101 (from preparation A) were raised on Millipore filters and stored frozen as described by Hanahan and Meselson^{24,25}. To carry out the hybridisation translation assay, the number of bacterial clones (from preparation A) indicated in the table were inoculated individually on to agar plates, incubated for 24 h and washed off with medium. This suspension was used to inoculate 1-l cultures from which plasmid DNA was purified as described (method B in ref. 25). The hybrid Le cDNA (20 µg) was cleaved with *Hind*III, mixed with 12 µg Le poly(A) RNA, 5 ng [³²P]-labelled rabbit β-globin mRNA (5,000 c.p.m.) and 0.1 µg *Pst*I-cleaved rabbit globin cDNA plasmid (Z-pBR322(H3)/RcBG-4.13)²⁶ in 40 µl 80% formamide, 0.4 M NaCl, 10 mM PIPES buffer (pH 6.4) and 5 mM EDTA, and heated for 4 h at 56°C. After diluting to 1 ml with 0.9 M NaCl and 0.09 M trisodium citrate (pH 7) and adjusting to 4% formamide, the solution was filtered through a Millipore filter (13 mm diameter, 0.4 µm pore size). The filter was washed for 10 min at 37°C in 0.15 M NaCl, 0.015 M trisodium citrate and 0.5% SDS, and the RNA recovered by heating the filter for 5 min in 0.5 ml 1 mM EDTA, 0.5% SDS and 3 µg ml⁻¹ yeast RNA at 75°C. The RNA was purified by oligo(dT) cellulose chromatography, precipitated with ethanol and assayed for IF-mRNA activity. To determine IF-mRNA activity, the RNA sample (up to 3 µg) was dissolved in 1–3 µl 15 mM Tris-HCl (pH 7.5) and 88 mM NaCl, and injected into 30–60 *Xenopus laevis* oocytes²⁷ (50 nl per oocyte). Oocytes were incubated for 12–16 h in Barth's medium²⁸, homogenised in 0.5 ml 50 mM Tris-glycine buffer (pH 8.9) and the supernatant was assayed for IF. In later experiments (Table 2), incubation was for 24–48 h and the incubation medium was assayed for excreted IF²⁹. IF was determined by the vesicular stomatitis virus (VSV) plaque reduction assay³¹. All values are expressed in international units. Values marked with an asterisk were obtained by hybridisation to diazobenzoyloxymethyl (DBM)-bound DNA, as described in ref. 22. Underlined values are considered positive. The control values in parentheses were obtained by hybridisation to pBR322 DNA. All manipulations involving live *E. coli* HB101 or *E. coli* X1776 containing Le cDNA-pBR322 hybrids were carried out in P3 containment conditions as described in the NIH Recombinant DNA Research Guidelines.

β-lactamase gene, can be expressed as a fused protein¹⁷ or, in certain circumstances, as an independent polypeptide¹⁸.

We identified an IF cDNA clone by a mRNA hybridisation translation assay¹⁹. Hybrid plasmid was prepared from pools of 512 bacterial clones, and 20 µg of each plasmid pool were cleaved with *Pst*I, denatured and annealed with 12 µg crude Le poly(A) RNA. [³²P]-labelled globin mRNA and rabbit β-globin cDNA plasmid were added to monitor hybridisation and all subsequent steps. Hybridised RNA was recovered from the filters, purified and injected into oocytes to determine its IF-mRNA activity. Control hybridisations were carried out with pBR322. The overall recovery of β-globin mRNA activity was only about 5% of the input.

Four out of 12 groups of 512 clones (δ, A, I and N) gave positive results by this assay, albeit erratically; controls were consistently negative in these groups (Table 1). However, in later experiments, controls occasionally gave a positive result, perhaps due to insufficient washing of the filters. A group of clones was scored as being positive if the value was higher than in the parallel control. The bacterial clones of group A were arranged in 8 subgroups of 64 each, and assayed as above. Three of these subgroups, A-II, A-III and A-IV, gave positive responses; the clones of A-III were regrouped into eight sets of eight.

The set A-III-4 was the first to yield a positive result; A-III-2 subsequently also gave positive results. DNA was prepared from the single A-III-4 clones and that from A-III-4C gave positive responses both by liquid and filter-bound hybridisation (Table 1). After recloning in *E. coli* HB101 the hybrid plasmid of clone A-III-4C, designated Z-pBR322(*Pst*I)/HcIF-4c (abbreviated to Hif-4c), was purified and cleaved with *Pst*I; it released a 320-base pair insert, that is, a fragment about one-third of the expected length of complete IF cDNA. The fragment bound IF mRNA efficiently (Table 2).

A set of colonies containing hybrid DNAs related to Hif-4c was identified by *in situ* hybridisation with [³²P]-labelled Hif-4c *Pst*I fragment. Among the 64 clones of A-III, three gave a strong hybridisation response, namely 4C, 2H and 7D, and two (1E and 3D) a weak one. A-III-2H had the largest insert, about 900 base pairs; it was recloned in *E. coli* HB101 and designated Z-pBR322(*Pst*I)/HcIF-2h (abbreviated to Hif-2h). In addition, 5,000 clones prepared as described, but using double-stranded Le-IF cDNA selected for length above 600 base pairs (preparation B, cloned in *E. coli* HB101), were screened by *in situ* hybridisation, using the same probe. Of 185 positive clones identified, 95 gave a strong and 90 a weak hybridisation response in the Grunstein-Hogness assay³⁰. The former were

designated *E. coli* HB101(Z-pBR322(*Pst*)/HcIF-SN1 to -SN95) (abbreviated to SN1 to SN95).

Properties of plasmid Hif-2h

The insert of plasmid Hif-2h, released by *Pst*I cleavage, was attached to diazophenylthioether (DPT) paper and the kinetics of hybridisation to IF mRNA in conditions of DNA excess determined (Fig. 1). In optimal conditions, about 7% of the IF-mRNA activity and 12% of the β -globin-mRNA activity were recovered, as measured in the oocyte system. Thus, the insert of Hif-2h hybridises to IF mRNA with about the same efficiency as does β -globin cDNA to β -globin mRNA.

Restriction and sequence analysis of Hif-2h (M. Schwarzstein, N. Mantei and M.S., unpublished results) showed that the insert has 910 base pairs of which 23 are 5'-terminal and 15 are 3'-terminal GC pairs; there is one site each for *Bsp*I (85), *Bgl*II (335) and *Eco*RI (710) endonucleases, two sites for *Pvu*II (125, 425), and three sites for *Ava*II (190, 385, 655) and none for *Hha*I, *Taq*I, *Hind*III, *Hpa*II, *Pst*I and *Bam*HI. (The values in parentheses indicate the distance in base pairs from the *Pst*I terminus corresponding to the 5' end of the mRNA.) The orientation of the cDNA insert, as ascertained by nucleotide sequence analysis, was such that the reading direction of the IF cDNA coincided with that of the β -lactamase gene.

Detection of IF activity in *E. coli* strains transformed with Hif-4c-related hybrid plasmids

The isolated Hif-2h *Pst*I fragment was joined to *Pst*I-cleaved pBR322 and to three plasmids, pKT279, pKT280 and pKT287, derived from pBR322 by deletions in the β -lactamase gene; DNA ligated into the *Pst*I site of this set can be translated in the three possible reading frames by readthrough from the β -lactamase sequence (K. Talmadge, personal communication). *E. coli* HB101 strains transformed with these hybrid DNAs were *E. coli* HB101 (Z-pBR322(*Pst*)/HcIF-2h-AH1 to -AH4), *E. coli* HB101 (Z-pKT279(*Pst*)/HcIF-2h-AH1 to -AH8) and so forth, or in abbreviated form, 322-AH1 to -AH4, 279-AH1 to -AH8, and so on. S-30 or S-100 extracts, from 24 of the AH and 49 of the SN strains grown to stationary phase, were tested for IF activity. The original Hif-2h-containing strain and many of the AH strains showed IF activity; three of them, 279-AH8, 280-AH3 and 287-AH6, were selected for further testing. Of

Table 2 Characterisation of the insert of hybrid plasmid Hif-4c by the mRNA hybridisation translation assay

DNA fragment	Amount of leukocyte poly (A) RNA (μ g)	Time of hybridisation (h)	IF activity (units ml ⁻¹)
Hif-4c	2.5	16	250: 100
β -globin cDNA	2.5	16	4: 1
Hif-4c	7.5	16	3,000: 1,000
β -globin cDNA	7.5	16	4: 30
Hif-4c	7.5	5	1,000: 1,000
β -globin cDNA	7.5	5	10: 1

The insert of plasmid Hif-4c was excised with *Pst*I, purified by electrophoresis through a 2% agarose gel and recovered by successive adsorption to and elution from hydroxyapatite and DEAE cellulose. 120-ng fragments were linked to each 0.25 cm² DPT paper (B. Seed, personal communication). Pre-hybridisation, hybridisation and elution of RNA were as described elsewhere¹¹. The RNA was injected into oocytes and IF activity was determined after 48 h by the cytopathic effect reduction assay¹⁰ (see Table 3 legend).

the 49 SN strains, 16 had IF activity; two of the highest producers, SN35 and SN42, and a negative control, SN32, were further examined. Table 3 shows the results obtained with S-100 extracts of log phase bacteria. IF activities ranged from 100 to 1,000 units per ml of S-100 extract derived from a 20-ml resuspension of the 2.0 g (approximately) of bacterial cells contained in 1 l of culture.

Characterisation of the IF activity produced in transformed *E. coli*

We tested the sensitivity of the IF activity to a protease by incubating S-100 extracts of 287-AH6 and SN35 for 30 min at 37°C with increasing amounts of trypsin. As a control, authentic human Le-IF was mixed with the (inactive) S-100 extract of SN32 (to give a similar protein concentration, 6 mg ml⁻¹) and digested in parallel. In all cases, the activity was partially abolished at 200 μ g ml⁻¹ and completely abolished at 1 mg ml⁻¹ trypsin.

Table 3 IF activity in extracts of transformed *E. coli*

S-100 extracts of <i>E. coli</i> HB101 transformed by:	IF activity (units per ml extract)
a Z-pBR322(<i>Pst</i>)/HcIF-2h	100: 100
b Z-pKT279(<i>Pst</i>)/HcIF-2h-AH8	100: 300
c Z-pKT280(<i>Pst</i>)/HcIF-2h-AH3	1,000: 1,000
d Z-pKT287(<i>Pst</i>)/HcIF-2h-AH6	200: 200
e Z-pBR322(<i>Pst</i>)/HcIF-SN35	1,000: 1,000
f Z-pBR322(<i>Pst</i>)/HcIF-SN42	300: 100
g Z-pBR322(<i>Pst</i>)/HcIF-SN32	0: 0

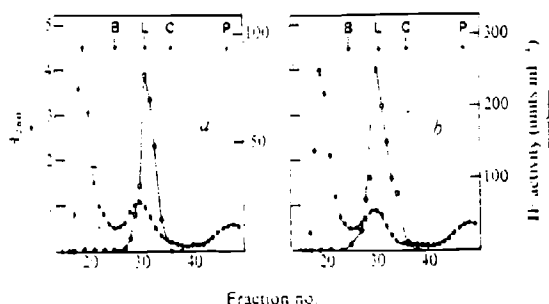


Fig. 2 Chromatography of Le-IF and *E. coli* IF on Sephadex G-100. (a) 0.9 ml S-100 extract of 280-AH3 (500 units, 6 mg protein ml⁻¹) and (b) 0.9 ml of a dilution of human Le-IF (1,000 units of preparation P-IF (ref. 34)) in S-100 extract of SN32 (no IF activity, 6 mg ml⁻¹) were mixed with cytochrome c (0.2 mg) (C), ¹²⁵I-phosphate (10³ c.p.m.) (P) and ¹²⁵I-labelled β -lactoglobulin (2.5 \times 10³ c.p.m.) (L) and chromatographed on a 0.9 \times 49-cm column of Sephadex G-100 in PBS at 4°C. Fractions of 0.7 ml were collected at 2.3 ml h⁻¹. IF activity was measured by the cytopathic effect reduction assay, and radioactivity, A₂₈₀ and A₄₁₀ (cytochrome c) were determined for each fraction. The position of bovine serum albumin (B) was determined in a separate run, relative to C and P.

The IF-cDNA insert of Hif-4c, excised with *Pst*I and purified as described in Table 2 legend, was joined to *Pst*I-cleaved pKT279, pKT280 and pKT287, respectively. *E. coli* HB101 was transformed with these products and tetracycline-resistant colonies were screened by *in situ* hybridisation¹² as described by Hanahan and Meselson¹³, using the Hif-4c *Pst*I fragment nick-translated with [α -³²P]dATP (1,100 Ci mmol⁻¹, NEN) and [α -³²P]dCTP (470 Ci mmol⁻¹, NEN) as labelled substrates¹⁴. Three clones (b-d) were selected in preliminary assays for IF activity. Clones c-g were from a set of IF-cDNA-containing clones identified among 5,000 *E. coli* HB101 transformed with Le-cDNA (preparation B, see Table 1 legend) by *in situ* hybridisation as above. e and f were shown to produce IF in a preliminary screening, and g was chosen as negative control. One-litre cultures of transformed *E. coli* were grown to an A₆₀₀ of about 0.8. The cells (about 2.0 g) were collected, washed with 50 mM Tris-HCl (pH 8), 30 mM NaCl and resuspended in 20 ml of the same buffer. Lysozyme was added to 1 mg ml⁻¹, after 30 min at 0°C, the suspension was frozen and thawed five times and centrifuged at 10,000 r.p.m. for 20 min. The supernatant was centrifuged at 40,000 r.p.m. for 1 h in a Spinco 60 rotor. The S-100 supernatants (about 6 mg ml⁻¹ protein in all cases) were assayed in duplicate by the cytopathic effect reduction assay and their IF content estimated relative to a standard IF preparation. IF activity was determined by the cytopathic effect reduction assay as follows. The IF samples, serially diluted 1:3 were mixed with 10³ CCL23 cells in the wells of a microtitre plate (Cooke) in MEM-10% newborn calf serum. After 24 h the medium was replaced by an appropriate dilution of Mengo virus in the same medium. 24 h later the medium was replaced with 0.5% crystal violet, 3% formaldehyde, 30% ethanol and 0.17% NaCl for 15 min; the wells were then washed exhaustively with water.

Table 4 Antibody titres of anti-Le-IF and anti-F-IF measured against different IF preparations

	Le-IF	F-IF	S-100 <i>E. coli</i> extracts	
			SN35	280-AH3
Sheep anti-Le-IF	100,000	3,000	30,000	30,000
Goat anti-F-IF	<10	1,000	<10	<10

About 10 units of each IF preparation were incubated for 1 h at 20 °C with different antiserum dilutions and the IF activity was determined by the VSV plaque reduction assay²³. The titres are given as the reciprocal of the highest dilution which raises the plaque counts by a factor of 2. The sheep anti-Le-IF was prepared as described elsewhere²³. Human Le-IF was purified to the P-IF stage²⁴.

Human Le-IF is stable at pH 2 (ref. 21). S-100 extracts of 280-AH3 and SN35, as well as 250 units human Le-IF mixed with (inactive) S-100 extract of SN32, were dialysed overnight against 0.1 M NaCl and 50 mM glycine-HCl (pH 2) buffer and then 5 h against phosphate-buffered saline (PBS). A precipitate was removed by centrifugation and the supernatant assayed by both cytopathic effect reduction and plaque reduction. In all cases the initial IF activity was recovered in full.

To compare the MWs of authentic Le-IF and the IF activity in transformed *E. coli* (*E. coli* IF), S-100 extracts were chromatographed on Sephadex G-100 columns. IF activity moved with a K_{av} of 0.46 in the case of 280-AH3 (Fig. 2a), 281-AH6 and SN35 (data not shown), which was slightly slower than authentic Le-IF (mixed with S-100 extract of SN32, Fig. 2b); the difference may, however, not be significant.

We compared the serological properties of authentic IFs and *E. coli* IFs. As shown in Table 4, sheep anti-human Le-IF had a similar titre against Le-IF and *E. coli* IF of SN35 and 280-AH3, and was 1/10th as active on fibroblast IF (F-IF); goat anti-human F-IF was active only against F-IF. Thus, *E. coli* IF is immunologically similar to Le-IF, and quite distinct from F-IF.

Both authentic Le-IF and *E. coli* IFs show specificity in regard to the cells on which they will act: they are most active on human cells, less active on monkey and mouse cells and inactive on chick cells (Table 5). It is not clear whether the relatively high activity of *E. coli* IF on monkey cells is significant; further experiments with the purified material are necessary.

As shown by Kerr^{4,5} and others, treatment of cells with IF increases 10- to 15-fold their level of oligoadenylate synthetase, an enzyme that condenses ATP to ppp(A2'p)_n5'A ($n = 1-4$). Cells were treated with various IF preparations and the cell extracts assayed by measuring the ³H radioactivity transferred from ³H-ATP to the dephosphorylation products of pppA2'p5'A and ppp(A2'p)₃5'A, namely (2'-5')ApA and (2'-5')ApApA. As shown in Table 6, (2'-5')ApA radioactivity was six- to ninefold higher in cells treated with Le-IF or *E. coli* IF than in controls treated with an inactive *E. coli* extract, and (2'-5')ApApA radioactivity was more than 14-33 times higher than in controls; there was no significant difference between the activity of Le-IF and S-100 extracts of 280-AH3 and SN35.

Table 5 IF activities measured on different cell types

Cells Expt 1	Le-IF	Interferon activity		
		F-IF	<i>E. coli</i> S-100 extracts	
			280-AH3	SN35
Human U amnion	6,000	2,000	600	600
Monkey Vero	600	400	350	350
Monkey GMK	350	200	350	110
Primary chick embryo fibro- blasts	<20	<20	<20	<20
Expt 2	Le-IF	mouse-IF	<i>E. coli</i> S-100 extracts	
			287-AH6	SN35
Human CCL23	1,000	ND	300	1,000
Mouse L929	40	120	40	120

Human Le-IF was preparation P-IF (ref. 34), mouse IF was the NIH standard. U cells were maintained by K.C. All cells were challenged with VSV, except for CCL23 cells, where Mengo virus was used. Experiment 1 was assayed by plaque reduction, experiment 2 by the cytopathic effect reduction assay. ND, Not done.

Thus, by all criteria tested, *E. coli* IF is very similar to authentic Le-IF, although, of course, the molecular structure may well differ in various respects.

Discussion

A strain of *E. coli* containing IF-cDNA was identified by an IF-mRNA hybridisation translation assay in which DNA from successively smaller pools of strains was screened. Because only 4 of 12 groups of 512 clones had originally given a positive response, we were surprised to find 5 IF clones in a selected group of 64. It is probable that, when used on large pools, the assay was at borderline sensitivity and only detected groups and subgroups particularly rich in IF-cDNA clones. The subsequent screening of 5,000 colonies using an IF-cDNA probe revealed 185 positive clones, a frequency of about 1:27. Taking into account the fact that the poly(A) RNA used to generate the clones had been enriched about 10-fold with respect to IF mRNA, the proportion of IF mRNA in poly(A) RNA from induced leukocytes was not less than 1:270.

The identification of the 910-base pair insert in Hif-2h as a cDNA copy of human Le-IF rests on two lines of evidence: (1) its capacity to hybridise selectively to IF mRNA, and (2) its ability to direct the synthesis, in *E. coli*, of a polypeptide with the

Table 6 Levels of oligoadenylate synthetase in human cells treated with Le-IF or *E. coli* IF

Cells treated with:	Cell protein (μg)	³ H-A in oligoadenylate (% of recovered radioactivity)	
		ApA	ApApA
1. S-100 extract of SN35 (200 units IF ml ⁻¹)	7.6	1.4	<0.1
	38	5.2	1.4
2. S-100 extract of 280-AH3 (200 units IF ml ⁻¹)	7.6	1.5	0.1
	38	7.8	3.3
3. S-100 extract of SN32 (no IF)	7.6	<0.1	<0.1
	38	0.9	<0.1
4. Le-IF (P-IF) (200 units IF ml ⁻¹)	7.6	1.3	0.25
	38	8.0	2.1

Confluent CCL23 cell monolayers in 50-mm dishes were treated with a mixture of 1 ml *E. coli* S-100 extract and 4 ml minimal essential medium (MEM)-10% newborn calf serum or a dilution of Le-IF (P-IF) in 5 ml medium. After 20 h the cells were lysed and supernatants prepared as described elsewhere¹³. Varying amounts of lysate were adsorbed to poly(U³H)-Sephacel and incubated as described elsewhere¹³, except that ³H-ATP (specific activity 40 Ci mmol⁻¹) was used instead of ³²P-ATP. After treatment with bacterial alkaline phosphatase, the products were separated by electrophoresis using ApA and ApApA as markers¹³. The paper was cut into strips and the radioactivity determined by scintillation counting. Most radioactivity was recovered in adenosine. 100% radioactivity was 3-5 × 10⁴ c.p.m.

biological activity of IF. The polypeptide has properties of human Le-IF in that it induces a virus-resistant state in human cells, to a lesser extent in monkey and mouse cells and not in chick cells, and is neutralised by antibody to human Le-IF but not to human F-IF. Moreover, *E. coli* IF stimulates the activity of isoenzyme synthetase in human cells to the same extent as does authentic Le-IF.

The IF-cDNA plasmids were constructed to allow synthesis of an IF molecule fused to part of β-lactamase. It seems likely, however, that the biologically active material is a non-fused polypeptide, because its formation is directed by hybrids derived from each of the three pKT plasmids and is therefore independent of the reading frame resulting from the construction. Moreover, a fused β-lactamase fragment should contribute 180 amino acids when the IF cDNA is inserted in the *Pst*I site of pBR322, but not more than 26 or 29 amino acids when it is linked to pKT280 or pKT287 (K. Talmadge, personal communication); in fact, there is no detectable difference in the size of the biologically active IF polypeptides made by the three strains. At the structural level, *E. coli* IF probably differs from authentic Le-IF by the absence of appropriate glycosylation. Also, it is possible that *E. coli* IF consists of the Le-IF sequence preceded by a signal sequence, as nucleotide sequence analysis of the cloned IF cDNA revealed a region coding for 22 amino

acids which follows the first AUG and precedes the stretch coding for mature IF (M. Schwarzstein, N. Mantei and M.S., unpublished results).

We do not know whether *E. coli* IF has the same specific activity as authentic Le-IF. If this were the case, the amount of active IF produced in transformed *E. coli*, about 20,000 units per l of culture, would correspond to one to two fully active molecules per cell. This would be consistent with the occurrence of rare translational events at the physiological initiation site of the IF sequence, and appropriate modifications of the hybrid plasmid should allow a considerable increase in the yield of active IF. If, however, lack of appropriate glycosylation diminishes the activity of the molecule, we shall have a problem on our hands.

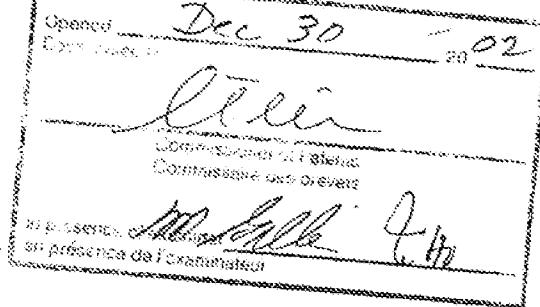
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Note added in proof: Taniguchi and his colleagues have recently identified by nucleotide sequence analysis (T. Taniguchi, personal communication) a fibroblast IF cDNA hybrid prepared from induced fibroblast poly(A) RNA and selected by hybridisation procedures (T. Taniguchi *et al.*, *Proc. Jap. Acad.* **55**, Ser. B, 464-469 (1979)).

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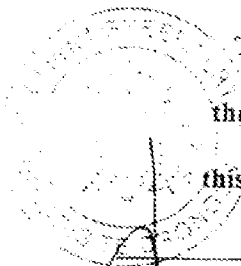
Human leukocyte and fibroblast interferons are structurally related.

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SUGANO EXHIBIT 1006
FIERS V. SUGANO
INTERFERENCE NO. 105,661



This is EXHIBIT FIERS-40
to
the Affidavit of Walter C. Fiers
sworn before me
this 30th day of November, 2001

Commissioner for Oath or Notary Public

SUMMARY

The coding sequences of the cDNAs of cloned human leukocyte interferon I and human fibroblast interferon show homologies of 45% at the nucleotide and 29% at the amino acid level. We estimate that the two genes were derived from a common ancestor about 500 to 1'000 million years ago.

The acid-stable human interferons are subdivided into two major groups, namely fibroblast interferons (F-IF) and leukocyte interferons (Le-IF); these are the major components of the interferons produced by induced fibroblasts and leukocytes, respectively. Some cells, such as the lymphoblastoid Namalva cell line, produce a mixture of 90% Le-IF and 10% F-IF (1, 2). The two interferon types have several features in common: both are glycoproteins with molecular weights ranging from 16'000 to 26'000 (3-9), the induction and shut-off of their synthesis appears to be under similar control (6), and at least some of the responses elicited in target cells are similar, such as induction of an antiviral state, which is accompanied by increased synthesis of several proteins (10-13). Nonetheless, the two kinds of interferons differ in many respects. Antibodies directed against Le-IF do not neutralize F-IF and vice-versa (14), the target cell specificities of the two IFs differ (15), and the sequences of the 13 amino terminal amino acids of F-IF and of Le-IF (from lymphoblastoid cells) show no homology (16, 22). Although Le-IF and F-IF are encoded by different mRNA species (17), it is not known whether these mRNAs are transcribed from distinct genes or whether they arise from the same gene via a common precursor which is processed or spliced in different modes.

We have recently cloned and sequenced one species each of Le-IF (Le-IF 1) (18,19) and F-IF cDNA (20,21). A second

species of Le-IF (Le-IF II) cDNA has recently been identified (M. Streuli, S. Nagata and C. Weissmann, unpublished results).

In Fig. 1 the nucleotide sequences of Le-IF I and F-IF cDNA were aligned so that the AUGs closest to their 5' termini coincided. From the amino terminal sequence published for F-IF (16) and lymphoblastoid Le-IF (22) one can determine that in the case of F-IF the 21st codon following the initiation triplet and in the case of Le-IF the 23rd codon represents the first amino acid of the interferon polypeptide. Presumably the stretch in between encodes a signal peptide. Since the putative signal peptide of Le-IF comprises 23 and that of F-IF 21 amino acids, the IF polypeptides, as aligned in Fig. 1, are shifted by two residues relative to their termini. In this alignment, 48 of 166 positions (29%) of the interferon polypeptides have identical amino acids. To plot the degree of homology between the F-IF and Le-IF as function of the map distance, the sequence was subdivided into segments of 8 amino acids (or 24 nucleotides), each overlapping by 4 amino acids (or 12 nucleotides) with the neighboring segments, and the percent coincidence of amino acids (and nucleotides) for each segment was determined (cf. van Ooyen et al., ref. 23). As seen in Fig. 2, amino acid sequences show three domains of homology. The first one, with the least degree of homology, corresponds to the putative signal sequence, which is rich in hydrophobic residues and has 4 identical amino acid positions out of 21; the second domain, between the 28th and 80th amino acid (counted on the Le-IF sequence), has 21 identical residues out of 51 (41% homology) and the third, between

positions 115 and 151 (Le-IF sequence), has 19 out of 35 identical residues (54%). The longest stretches of contiguous conserved amino acids are Gln-Phe-Gln-Lys (pos. 47-50 of Le-IF and 49-52 of F-IF) and Cys-Ala-Trp (pos. 139-141 and pos. 141-143, respectively). The latter sequence is notable because it comprises Cys and Trp, which are preferentially conserved in related proteins (24). Table 1 shows that conservation was highest between the interferon polypeptides (not considering the signal sequences) for Trp, Phe, Arg, Cys and Tyr residues, in agreement with the general experience that the amino acids most likely to be conserved between related proteins are Trp > Cys > Tyr > Arg > Phe, His (24). Even where amino acids are conserved, the codons show one or more nucleotide changes in half the instances. The codons of 3 out of 7 conserved Leu residues are non-related, as are 2 of 4 codons pertaining to conserved Ser residues. This suggests that there is a strong selective pressure favoring the conservation of several amino acids. It is quite likely that at least some of the conserved amino acids are essential for a function common to Le-IF and F-IF, perhaps the induction of the virus-resistant state in the target cell. These findings may provide guidelines for the tailoring of modified (25), possibly shorter polypeptides possessing certain activities of interferon.

The nucleic acid sequences show an average homology of 43% in the domain of the signal sequence and of 45% in the interferon polypeptide sequence. On a random basis, about 25% of the nucleotide positions should

coincide. Within the interferon coding sequence, the nucleotide homologies are more evenly distributed than the amino acid homologies. However, one may distinguish, albeit to a less pronounced degree, the same two blocks of similarity noted for the amino acids. The longest region without mismatches extends for 13 nucleotides (cf. 47th to 51st codon of Le-IF vs. 49th to 53rd codon of F-IF). There are, in addition, sequences of 17, 18 and 20 nucleotides with 3, 3 and 4 mismatches, respectively. The heteropolymeric 3'-terminal non-coding region of Le-IF cDNA has 242 nucleotides, and is longer by 39 residues than its counterpart in F-IF cDNA. In aligning the two sequences four gaps were introduced to maximize homology, as described by van Ooyen et al.⁽²³⁾ Thereby, several segments were matched with 29 to 41% homology. The introduction of gaps in the alignment may be justified in view of the arguments presented previously, that introns and non-coding regions of reduplicated genes diverge as a consequence of block insertions and/or deletions in the course of evolution (23,26).

It is unlikely that the extent of homology between Le and F-IF cDNA would allow meaningful crosshybridization between the two species.

On the basis of our findings there is no doubt that Le-IF and F-IF genes are derived from a common ancestral sequence. When did the separation of these genes occur? Human α and β globin show 57% amino acid mismatches, and human β -globin and myoglobin, as well as α -globin and myoglobin, 76% mismatches.

If the rate of divergence of interferons and globins is comparable (however, cf. p. 50, ref. 24, for proteins showing both higher and lower rates) then the separation of interferon genes occurred after that of myoglobin and hemoglobins and before that of α - and β -globins, i.e. between 500 and 1000 million years ago (24). The interferon genes may thus be about as old as the vertebrates (27).

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TABLE 1 Conservation of amino acids in leukocyte and fibroblast interferon.*

	F-IF	Le-IF	Conserved amino acids	Number of changes in codon of conserved amino acids			
				0	1	2	3
Leu	25	22	8	1	4	3	
Cys	3	5	2	1	1		
AsN	12	6	1	1			
Arg	11	12	5	1	3	1	
Phe	9	8	4	2	2		
Pro	1	6	1		1		
Gln	11	10	3	3			
Lys	11	8	3	2	1		
Ala	6	10	2	2			
Glu	13	15	4	4			
Ile	11	7	3	2	1		
Ser	9	13	4		2	1	1
Trp	3	2	2	2			
Tyr	10	4	4	1	3		
Val	5	6	1	1			
Asp	5	11	1	1			
Thr	6	9	0				
Gly	6	3	0				
Met	4	6	0				
His	5	3	0				
	<u>166</u>	<u>166</u>	<u>48</u>	<u>24</u>	<u>18</u>	<u>5</u>	<u>1</u>

*The data are from Taniguchi et al. (ref. 21) and Mantei et al. (ref. 19).

FIGURE LEGENDS

Fig. 1 Comparison of the nucleotide sequences of human leukocyte interferon I (Le-IF I) and human fibroblast interferon cDNA and of the derived amino acid sequences. The sequences are from Mantei et al. (19) and Taniguchi et al. (21). They were aligned to give maximal homology. Identical amino acids are framed, identical nucleotides are marked by a dot. S1 to S23 indicate the amino acids of the putative signal sequence; 1 to 166 the amino acids of the interferon polypeptides.

Fig. 2 Similarity of the nucleotide and amino acid sequences of human leukocyte interferon I and fibroblast interferon. The sequences shown in Fig. 1 were subdivided in segments of 8 amino acids or 24 nucleotides, each overlapping by 4 and 12 residues, respectively, with the neighboring segments. The percentage of coincident residues was plotted as a function of map position. Open vertical blocks, nucleotides; filled vertical blocks, amino acids. L-IF, leukocyte interferon cDNA; F-IF, fibroblast interferon cDNA; lines, non-coding sequences; hatched bars, putative signal peptide; open bars, interferon polypeptide.

Fig. 1

23
 G CT CTA GGT TCA GAG TCA CCC ATC TCA GCA AGC CCA GAA GTA TCT GCA ATA TCT ACG ATG GCC TCG CCC TTT
 S1
 MET ALA SER PRO PHE
 MET THR ASN LYS CYS
 GTC AAC ATG ACC AAC AAG TGT

S10 S20
 1
 GCT TTA CTG ATG GTC CIG GIG GTG CTC AGC TGC TCT CIG GGC TGT GAT CTC CCT GAG ACC
 ALA LEU LEU MET VAL LEU VAL VAL LEU SER CYS LYS SER SER CYS SER LEU GLY CYS ASP LEU PRO GLU THR
 LEU LEU GLN ILE ALA LEU LEU LEU CYS PHE SER THR THR ALA LEU MET SER TYR ASN LEU LEU GLY PHE
 CTC CTC CAA ATT GCT CTC CIG CTC TIG TGC TTC TCC ACT ACA GCT CTT TCC AIG ACC TAC AAC TIG CTT GGA TTC
 1

10
 CAC AGC CTG GAT AAC AGG AGG ACC TTG ATG CTC CTG GCA CAA ATG AGC AGA ATC TCT CCT TCC TCC TGT CTG 30
 HIS SER LEU ASP ASN ARG ARG THR LEU MET LEU LEU ALA GLN MET SER ARG ILE SER PRO SER SER CYS LEU
 LEU GLN ARG SER SER ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR CYS LEU
 CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAT TGC CTC 30
 10 20 30

40
 ATG GAC AGA CAT GAC TTT GGA TTT CCC CAG GAG GAG GAG TTT GAT GGC AAC CAG TTC CAG AAG GCT CCA GCC ATC 50
 MET ASP ARG HIS ASP PHE GLY PHE PRO GLN GLU GLU GLU PHE ASP GLY ASN GLN PHE GLN LYS ALA PRO ALA ILE
 LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU GLN GLN PHE GLN LYS GLU ASP ALA ALA
 AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA 50
 40 50

60
 TCT GTC CTC CAT GAG GAG ATT CAG CAG ATC TTC AAC CTC TTT ACC ACA AAA GAT TCA TCT GCT GGT TGG GAT
 SER VAL LEU LEU HIS GLU LEU ILE GLN GLN ILE PHE ASN LEU PHE THR THR LYS ASP SER SER ALA ALA TRP ASP
 LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER THR GLY TRP ASN
 TTG ACC ATC TAT TAT GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT
 70
 80

80
 GAG GAC CTC CTA GAC AAA TTC TGC ACC GAA CTC TAC CAG CAG CAG CAG ATT GAC TTG GAA GCC TGT GTG ATG CAG
 GLU ASP LEU LEU ASP LYS PHE CYS THR GLU LEU TYR GLN GLN LEU ASN ASP LEU GLU ALA CYS VAL MET GLN
 GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU
 GAG ACT ATT GGT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTC GAA GAA
 90
 100

110

GAG GAG AGG GIG GGA GAA ACT CCC CTG ATG AAT GCG GAC TCC ATC TTG GCT GTG AAG AAA TAC TTG CGA AGA
 GLU GLU ARG VAL GLY GLU THR PRO LEU MET ASN ALA ASP SER ILE ILEU ALA VAL LYS LYS TYR PHE ARG ARG
 LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER ILEU HIS LEU LYS ARG TYR GLY ARG
 AAA CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC CTG AAA ACA TAT TAT GGG AGG
 110 120

120

130

ATC ACT CTC TAT CTG ACA GAG AAG AAA TAC AGC CCT TGT GCC TGG GAG GGT GTG AAG ACA GAA ATC ATG AGA
 ILE THR LEU TYR LEU THR GLU LYS LYS TYR SER PRO CYS ALA TRP GLU VAL VAL ARG ALA GLU ILE MET ARG
 ILE LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG
 ATT CTG CAT TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA AGG
 130 140 150

140

150

160
 166
 TCC CTC TCT TTA TCA ACA AAC TTG CAA GAA AGA TTA AGG AGG AAG GAA TAA CAT CTG GTC CAA CAT GAA AAC
 SER LEU SER LEU SER THR ASN LEU GLN GLU ARG LEU ARG ARG LYS GLU
 ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN
 AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC TGA AGA TCT CCT AGC CTG TGC CTC TGG GAC
 160 166

AAT TCT TAT TGA CTC ATA CAC CAG GTC ACG CTT TCA TGA ATT CTG TCA TTT CAA AGA CTC TCA CCC CTG CTA

T GGA CAA TTG CTT CAA GCA TTC TTC AAC CAG CAG AIG CTG TTT AAG TGA CTG ATG GCT AAT GTA

-IA ACT AIG ACC ATG CTG ATA AAC TGA YTT AYC IAY TTA AAT ATT IAT TTA ACT AIT CAT AAG AIT YAA ATT

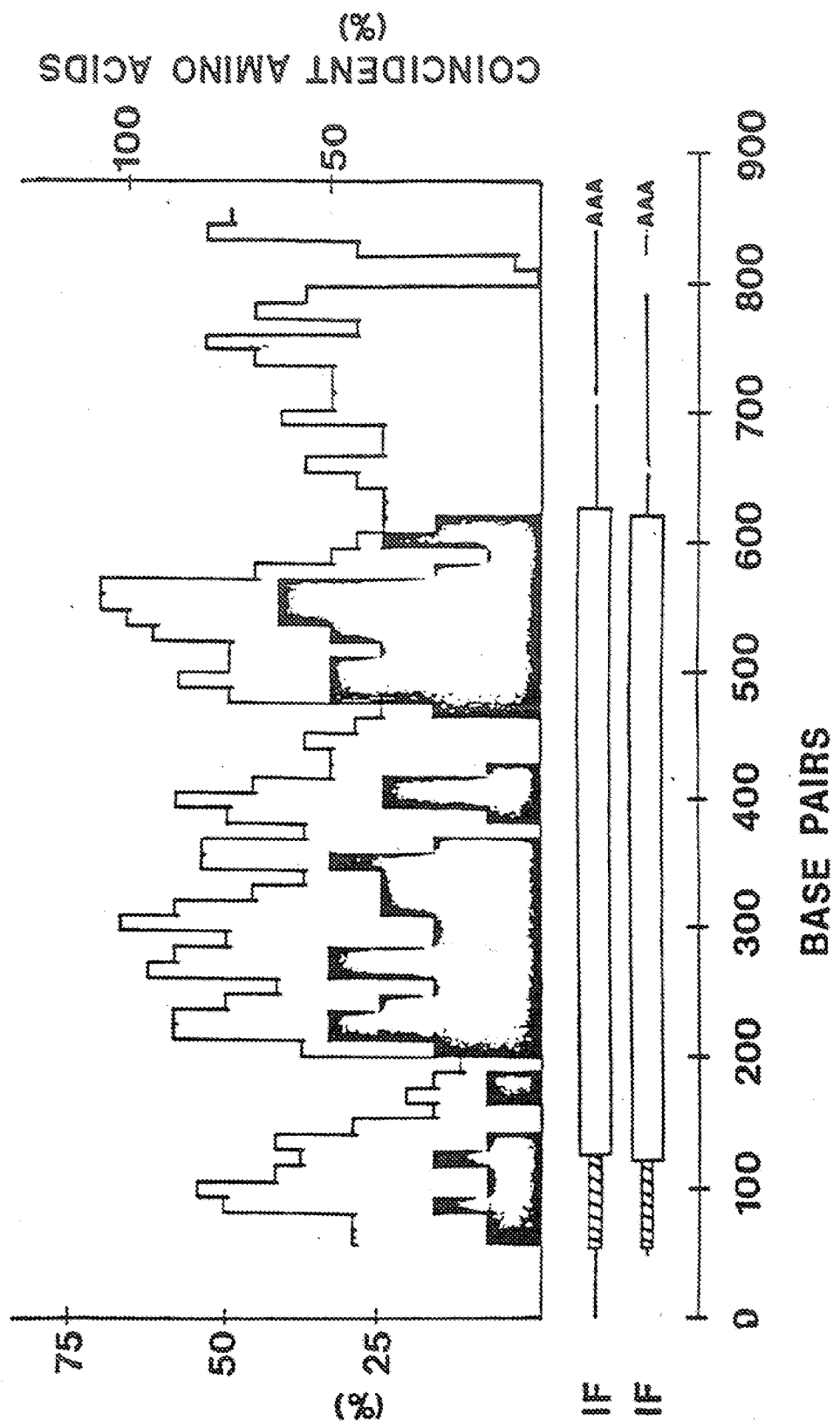
CIG CAT AIG AAA GGA CAC TAG AAG AIT TIG AAA YIT TTA TTA AAT IAT GAG TTA YIT TTA YIT AIT YAA ATT

ATT YTT GTT CAT ATA ACG TCA TGT GCA CCT TTA CAC TGT GGT TAG TGT AAT AAA ACA IGT TCC TTA TAT YTA

-----IT AIT ITG GAA AAT AAA TTA YIT ITG GIG CAA A-A

CTC AAA AAA A

CTC AAA A



Identification of the Translation Products of Human Fibroblast Interferon mRNA in Reticulocyte Lysates

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(Received December 28, 1978)

Messenger RNA was purified from human foreskin fibroblasts FS11, a high interferon-producer line, after induction with synthetic double-stranded RNA. The mRNA was translated in a cell-free protein-synthesis system from rabbit reticulocytes. The translation products, containing biologically active human interferon, were immunoprecipitated by a serum from rabbits immunized against partially purified interferon. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis in dodecylsulfate shows that the product of human fibroblast interferon mRNA is a 23000-*M*_r polypeptide. Methods are described for the synthesis and rapid identification of this polypeptide, which should be useful for structural analysis of interferon and isolation of its mRNA.

Interferon mRNA from human cells has been translated in heterologous intact cells [1,2], in *Xenopus laevis* oocytes [3,5-8] and in a variety of cell-free systems [3-5]. In all these cases, as found also for mouse interferon mRNA [9-14], biologically active interferons, showing the correct species specificity and immunological properties, were obtained. The translation of human fibroblast interferon mRNA was reported to be low in cell-free protein-synthesis systems as compared to oocytes [5]. Use of cell-free extracts, such as nuclease-treated reticulocyte lysates [15], would however be advantageous to study the proteins synthesized, because of the high efficiency and low background of this translation system. This work describes the identification by immunoprecipitation and polyacrylamide gel electrophoresis, of a highly labeled 23000-*M*_r polypeptide which appears as the specific product of human fibroblast interferon mRNA translation in reticulocyte lysates. This method should allow the structural analysis of this polypeptide and facilitate the purification of its mRNA.

MATERIALS AND METHODS

Growth of FS11 Cells and Induction of Interferon

Human foreskin fibroblast FS11 cell cultures were established in our laboratory by Dr D. Gurari-Rotman. These diploid cells, grown from foreskin

explants taken 8 days after birth, were selected among 15 individual isolates for their capacity to produce high titers of interferon. The cells were grown in Eagle's minimum essential medium with 10% fetal calf serum in 5% CO₂, 95% air at 37 °C and maintained by subculture at 1:5, in 90-mm plastic dishes. For interferon production [16], five such plates of cells were seeded in 4-pint (2.27-l) roller bottles with 80 ml minimum essential medium containing 10% fetal calf serum. Three days after confluency (8th day) the cultures were exposed to 100 µg/ml poly(rI)·poly(rC) and 50 µg/ml cycloheximide in 25 ml medium without serum. After 3.5-4 h actinomycin D was added (1 µg/ml) and 1.5-2 h later the cells were washed in NaCl/P_i and incubated at 34 °C with 25 ml minimum essential medium containing 13 mM Hepes and 6 mM tricine buffers pH 7.5 with 0.15% human serum albumin. After 18 h, the medium, which routinely contained 5-20 × 10⁴ U interferon/ml, was stored at -20 °C.

Measurement of Interferon Antiviral Activity

A rapid semi-automated micromethod was used to measure the reduction of viral RNA replication by interferon. Dilutions of interferon were made in 96-well microplates in 50 µl medium containing 10% fetal calf serum; 50 µl FS11 cells (obtained by Viokase treatment of 15-day-aged plates) were seeded at 25000 cells/well. After 18 h at 37 °C, the medium was removed and 50 µl of medium containing 2% serum, 0.5-1 × 10⁵ p.f.u. vesicular stomatitis virus (VSV) and 0.075 µg actinomycin D were added. After 25 µl medium containing [³H]uridine (50 Ci/mmol).

Abbreviations. Poly(rI)·poly(rC), poly(inosate)·poly(cytidylic acid) double-stranded RNA; NaCl/P_i, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; tricine, N-tris(hydroxymethyl)-methyl-glycine; p.f.u., plaque-forming units; vsv, vesicular stomatitis virus; CM, carboxymethyl.

Sugano Exhibit 1007
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Interference 105,661

EXHIBIT A

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explants taken 8 days after birth, were selected among 15 individual isolates for their capacity to produce high titers of interferon. The cells were grown in Eagle's minimum essential medium with 10% fetal calf serum in 5% CO₂, 95% air at 37 °C and maintained by subculture at 1:5, in 90-mm plastic dishes. For interferon production [16], five such plates of cells were seeded in 4-pint (2.27-l) roller bottles with 80 ml minimum essential medium containing 10% fetal calf serum. Three days after confluency (8th day) the cultures were exposed to 100 µg/ml poly(rI)·poly(rC) and 50 µg/ml cycloheximide in 25 ml medium without serum. After 3.5-4 h actinomycin D was added (1 µg/ml) and 1.5-2 h later the cells were washed in NaCl/P_i and incubated at 34 °C with 25 ml minimum essential medium containing 13 mM Hepes and 6 mM tricine buffers pH 7.5 with 0.15% human serum albumin. After 18 h, the medium, which routinely contained 5-20 × 10⁴ U interferon/ml, was stored at -20 °C.

Measurement of Interferon Antiviral Activity

A rapid semi-automated micromethod was used to measure the reduction of viral RNA replication by interferon. Dilutions of interferon were made in 96-well microplates in 50 µl medium containing 10% fetal calf serum; 50 µl FS11 cells (obtained by Viokase treatment of 15-day-aged plates) were seeded at 25000 cells/well. After 18 h at 37 °C, the medium was removed and 50 µl of medium containing 2% serum, 0.5-1 × 10⁵ p.f.u. vesicular stomatitis virus (VSV) and 0.075 µg actinomycin D were added. After 1 h, 25 µl medium containing 2% serum and 1.5 µCi [³H]uridine (50 Ci/mmol) were added and incubation

Abbreviations. Poly(rI)·poly(rC), poly(inosate)·poly(cytidylic acid) double-stranded RNA; NaCl/P_i, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; tricine, N-tris(hydroxymethyl)-methyl-glycine; p.f.u., plaque-forming units; vsv, vesicular stomatitis virus; CM, carboxymethyl.

continued for 6 h. The medium was removed and 50 μ l of 0.7% sodium dodecylsulfate were added, followed by 50 μ l of cold 20% trichloroacetic acid. The content of the microplate was automatically transferred to glass filters in a Dynatech Multimash where the filters were washed with 5 ml cold 10% trichloroacetic acid and ethanol. The dried filters were counted in a Tricarb scintillation counter. The 100% value was taken from wells receiving no interferon and varied between 10000 and 20000 counts/min. Wells without virus contain less than 500 counts/min. Fig. 1 shows a titration curve for a fibroblast interferon standard. The 50% reduction point was obtained at 1–2 U interferon/ml. The interferons used as standards were obtained from NIAID (GO23-902-527), from Dr J. Vilcek and from Dr E. Sulkowsky, the titers being established by reduction of the cytopathic effect of VSV. We used the reduction assay for some experiments as well, but, for most experiments we used the rapid radioactive assay described above. In some cases, a 24-well microplate was used and the procedure modified so that each well contained 120000 cells in 0.4 ml. To measure the anti-interferon titer of immune rabbit serum, serial dilutions of antiserum were added to a constant concentration of interferon (15–30 U/ml). The antiserum titer is the highest dilution which relieved by 50% the inhibition of [3 H]uridine incorporation caused by interferon. Controls of antiserum alone were included in the assays.

Antiserum to Partially Purified Human Fibroblast Interferon

About 10^7 U of crude FS11 interferon were loaded on 5–10-ml columns of sterilized CM-Sephadex

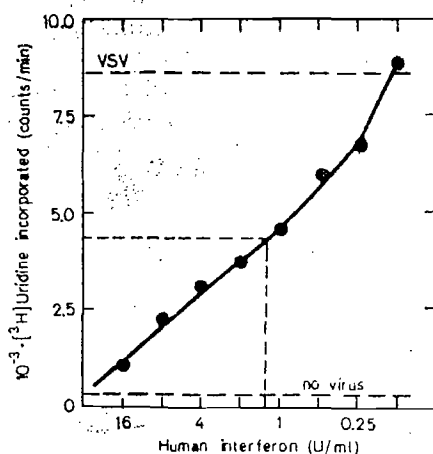


Fig. 1. Rapid radioactive assay of interferon. A standard solution of human fibroblast interferon (64 U/ml) was diluted 1:4 in the first well of a 96-well microplate and then serially diluted twofold. The final volume in each well was 0.1 ml. Incorporation of [3 H]uridine in VSV-infected FS11 cells was measured as detailed in Methods

equilibrated in 0.1 M sodium phosphate buffer pH 6.0. Over 90% of the proteins were not retained and interferon was eluted by a 0.1–0.75 M NaCl gradient in the same buffer. The specific activity of the interferon which eluted at about 0.35 M NaCl was above 10^7 U/mg protein. Rabbit serum albumin (0.1%) was added for stabilization and the material was concentrated by vacuum dialysis to 2×10^6 U/ml. After dilution 1–2-fold in complete Freund's adjuvant, 10^6 U interferon were injected subcutaneously to individual rabbits. After 6–10 injections at 2–3-week intervals, the animals were bled and the anti-interferon titer of the serum determined as above. In one rabbit, a titer of 100–200 U/ml was seen at 3 months and after 8 months the titer was 250–500 U/ml. Non-immune serum was taken from the same animals prior to immunization.

Preparation of mRNA from Poly(rI) · Poly(rC)-Induced and Non-Induced FS11 Cells

Batches of mRNA were prepared from between 10 and 40 4-pt (2.27-l) roller bottles. Cells were first exposed to 100 μ g/ml poly(rI) · poly(rC) and 50 μ g/ml cycloheximide for 3.5 h, then 1 μ g/ml actinomycin D was added and 4.5 h after the start of induction the cultures were rinsed twice with NaCl/ P_i . The cells were scraped into ice-cold NaCl/ P_i with a rubber policeman and spun down at $1600 \times g$ for 3 min. Cells were washed in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ and resuspended in the same buffer (1.5 ml/bottle) with 1% Nonidet P40 (NP-40). After 10 min on ice, the cell extracts were centrifuged at $1600 \times g$ for 3 min, adjusted to 200 mM Tris-HCl pH 9, 50 mM NaCl, 10 mM EDTA, 1.2 mM $MgCl_2$ and 0.5% sodium dodecylsulfate and extracted with an equal volume of 80% phenol, 12% cresol, 0.08% 8-hydroxyquinoline. Alternatively, cells were scraped and extracted with 4 ml/bottle of the phenol/cresol/8-hydroxyquinoline mixed with an equal volume of 4% sodium *p*-aminosalicylate according to Kirby [17]. After repeated extractions, the aqueous phase was made 0.2 M in sodium acetate pH 5 and 2 vol. ethanol were added. The precipitate was dissolved in 10 mM Tris-HCl pH 7.4, 0.5% sodium dodecylsulfate, heated 3 min at 65 $^{\circ}C$ and made 0.5 M in NaCl; the polyadenylated RNA was purified on oligo(dT)-cellulose [18]. 25 μ g polyadenylated RNA could be recovered from 10 bottles.

Translation of mRNA

Polyadenylated RNA was translated in a reticulocyte cell-free system. The reticulocyte lysate was prepared according to Gilbert and Anderson [19]. Endogenous mRNA was inactivated by treatment

with micro and Jackson final volume pH 7.6, 70 n 8 mM creati each of the 10 μ Ci of [3 S] ml rabbit li 0.1–0.5 μ g cubated at 3

Immunoprecipitation

Aliquots were added: interferon an of antiserum temperature, inactivated, fr bacteria [20] room temper plexes adsorl pelleted 2 mir twice with Na lation product resuspended i new tube and in 30 μ l of ele 3% sodium do 10% glycerol recentrifugatio was processed

Polyacrylamide Gel Electrophoresis

For total tr reaction mixtu sample buffer. the reaction mi agitated to pre liquid was remo P_i and the tube electrophoresis were prepared a 100 $^{\circ}C$ for 5 min amide gels [21]. (constant voltage subjected to fluo graphed with Ko

RESULTS

Antiviral Activity

Polyadenylate induced by poly(r tion was translate

with micrococcal nuclease as described by Pelham and Jackson [15]. The reaction mixture contained in a final volume of 25 μ l: 6 μ l of lysate, 20 mM Hepes pH 7.6, 70 mM potassium acetate, 0.3 mM spermidine, 8 mM creatine phosphate, 2 mM dithiothreitol, 25 μ M each of the protein amino acids except methionine, 10 μ Ci of [35 S]methionine (550 Ci/mmol), 80–160 μ g/ml rabbit liver tRNA, 4 μ g/ml creatine kinase and 0.1–0.5 μ g of mRNA. Reaction mixtures were incubated at 30 °C for 1 h.

Immunoprecipitation of Translation Products

Aliquots (e.g. 25 μ l) of the translation reaction were added to tubes containing an equal volume of interferon antiserum, or non-immune serum. Dilutions of antiserum were made in NaCl/ P_i . After 1 h at room temperature, 25 μ l of a 10% suspension of heat-inactivated, formaldehyde-fixed *Staphylococcus aureus* bacteria [20] was added and the suspension kept at room temperature for 1 h. Antigen-antibody complexes adsorbed to the inactivated bacteria were pelleted 2 min in a Microfuge (8000 \times g) and washed twice with NaCl/ P_i . To avoid contamination by translation products adsorbed to the tube walls, the pellet, resuspended in 50 μ l NaCl/ P_i , was transferred to a new tube and recentrifuged. The pellet was suspended in 30 μ l of electrophoresis sample buffer containing 3% sodium dodecylsulfate, 0.7 M 2-mercaptoethanol, 10% glycerol in 60 mM Tris-HCl pH 6.8, and after recentrifugation in the Microfuge, the supernatant was processed for gel electrophoresis.

Polyacrylamide Gel Electrophoresis

For total translation products, an aliquot of the reaction mixture was added into electrophoresis sample buffer. For tube-bound proteins, aliquots of the reaction mixture were added to Eppendorf tubes, agitated to produce maximum adsorption and all liquid was removed. The tube was washed with NaCl/ P_i and the tube-bound proteins dissolved by adding electrophoresis sample buffer. Immunoprecipitates were prepared as above. All samples were heated to 100 °C for 5 min and applied to 12% slab polyacrylamide gels [21]. Electrophoresis was for 3 h at 150 V (constant voltage). After electrophoresis, the gels were subjected to fluorography [22], dried and autoradiographed with Kodak SB-5 X-ray film.

RESULTS

Antiviral Activity of Translation Products

Polyadenylated-mRNA from FS11 cell cultures induced by poly(rI) + poly(rC) for interferon production was translated in reticulocyte lysates and [35 S]-

methionine incorporation into proteins was measured (Table 1). The titer of interferon produced *in vitro* was determined by measuring the reduction of [3 H]-uridine incorporation in VSV-infected FS11 cells, in comparison to known solutions of human fibroblast interferon, by the technique illustrated in Fig. 1. As shown in Table 1, a 0.1-ml translation reaction with 2 μ g mRNA from induced FS11 cells, gave a strong reduction of [3 H]uridine incorporation and an interferon titer of 250 U/ml, while the same reaction without mRNA had no antiviral activity. Comparing different batches of mRNA from induced cells, the interferon titer obtained varied with the mRNA-dependent [35 S]methionine incorporation into protein. Table 1 also shows that addition of polyadenylated mRNA from non-induced FS11 cells produced no antiviral activity, although the non-induced mRNA was as actively translated into proteins as the induced mRNA preparation.

Immunoprecipitation of Translation Products

Analysis of the [35 S]methionine-labeled translation products by polyacrylamide gel electrophoresis shows that a large number of polypeptides are formed in response to FS11 mRNA (Fig. 2, lane 1, 2). In the absence of exogenous mRNA, only traces of globin could be seen (not shown). The total translation products were reacted by the procedure described in Methods with serum from a rabbit immunized against a partially purified preparation of interferon from FS11 cells. The immunoglobulins were precipitated with protein-A – Sepharose, the Sepharose pellet was washed with electrophoresis sample buffer containing dodecylsulfate and the proteins recovered were submitted to electrophoresis on the polyacrylamide gel. Fig. 2 (lanes 3 and 4) shows that only a few polypeptides reacted with the immune serum. Comparison of the products of mRNA from non-induced and induced FS11 cells, shows that induced mRNA (lane 4) directs the synthesis of a prominent polypeptide, which is completely absent from the products of non-induced mRNA. The molecular weight of this polypeptide was estimated by comparison with known protein markers to be about 23000 (see also Fig. 3).

It is clear from Fig. 2 that antiserum of rabbits immunized with interferon partially purified on CM-Sephadex contains antibodies against several other polypeptides, but these are seen in the products of mRNA from non-induced FS11 cells as well. These antigens may be proteins normally secreted by the fibroblasts. Interestingly a polypeptide of M_r about 60000 was seen to be increased in the translation products of mRNA from induced FS11 but was not immunoprecipitated by the interferon antiserum.

When serum from a non-immunized rabbit was used instead of immune serum, the specific precipita-

Table 1. *Antiviral activity of translation products in vitro*

For experiment 1, translation reactions with 2 μ g mRNA in 0.1 ml were serially diluted and assayed for antiviral activity in a 24-well microplate in a volume of 0.4 ml/well. For experiment 2, a different set of translation reactions were assayed in a 96-well microplate with 0.1 ml/well. The reduction of [3 H]uridine incorporation in VSV-infected FS11 cells was measured as in Methods and Fig. 1. Data from individual dilution points are shown. Titers were calculated by comparison with standard solutions of human fibroblast interferon, similarly diluted. The [35 S]-methionine incorporation into protein is given for 2 μ l of each translation reaction assayed.

Expt	Conditions	Antiviral activity		Translation ([35 S]methionine incorporation)	
		[3 H]uridine incorporation at dilution of	titer		
		1:128	1:256		
		counts/min	U/ml	counts/min	
1.	Translation reaction:				
	without mRNA	28 830	28 300	0	5 500
	with induced mRNA	4 450	18 450	250	54 900
	Interferon standard:				
	500 U/ml	3 600	10 864		
	No addition	27 000			
		at 1:32 dilution			
		counts/min			
2.	Translation reaction				
	without mRNA	9 950		1 675	
	with non-induced mRNA	9 800	0	25 400	
	with induced mRNA	5 700	60	29 450	
	Interferon standard:				
	125 U/ml	3 650			
	250 U/ml	1 730			

tion of the 23 000- M_r band did not occur (Fig. 3). This was verified with immune and non-immune serum from several individual rabbits. The antisera used for immunoprecipitation were assayed for their ability to neutralize the biological activity of interferon. The anti-interferon titer was expressed as the highest dilution of antiserum which reduces by 50% the antiviral effect of 1.5–3 U of interferon in 0.1 ml. Antiviral activity was measured in 96-well microplates by the reduction of [3 H]uridine incorporation in VSV-infected FS11 cells, as described in Methods. Fig. 4 shows the correlation between the anti-interferon titer and the ability of the antiserum to precipitate the 23 000- M_r polypeptide product from the cell-free translation products. The antiserum with the highest interferon-neutralizing titer was also the best for immunoprecipitation, while antisera which did not neutralize interferon's biological activity did not precipitate the 23 000- M_r polypeptide. No other polypeptide on the autoradiographs showed such a correlation. With a serum of anti-interferon titer 250–500 U/ml, the half-maximum precipitation of the 23 000- M_r polypeptide was seen at a serum dilution of 1:50 (Fig. 3, lane 6). Little precipitation was seen at 1:250 and optimal precipitation was seen at 1:10.

Properties of the 23 000- M_r Polypeptide and of Its mRNA

The immunoprecipitated 23 000- M_r band has the size of human fibroblast interferon. Electrophoresis of CM-Sephadex-purified interferon produced by cultures of FS11 cells is shown in Fig. 5. Slices from the gel were extracted in the presence of serum proteins and assayed for their antiviral activity. In comparison with known markers, the molecular weight of the active interferon was found to be about 24 500. The size of the product *in vitro* (Fig. 3), which appears only when mRNA from induced FS11 cells and when immune interferon antiserum are used, is therefore very close to the size of interferon *in vivo*. The small difference (about 6%) could be due to experimental errors or to post-translational modifications (see Discussion). Extraction of dodecylsulfate gel electrophoresis of the translation products *in vitro* by the same procedure as above, showed that some biological activity comigrates with the 23 000- M_r polypeptide band. Over 10^5 counts/min of 35 S-labeled polypeptide could easily be prepared in this way.

During our early attempts to immunoprecipitate the cell-free product, we observed that the 23 000- M_r

Fig. 2. *Translation products* [35 S]methionine polyadenylated FS11 cells analyzed for interferon activity. Interferon A — was washed 5 min and analyzed for total cytoplasmic gel in dodecyl sulfate gels. The results were = 13 000 was fluor band

polypeptide of glass translation titer about 1 hot-acid the tube various radioactivity only hydrolyzed prevent allows to Fig. 6 (lane 1) the 23 000 mRNA lane NI) at the end be recovered has severe

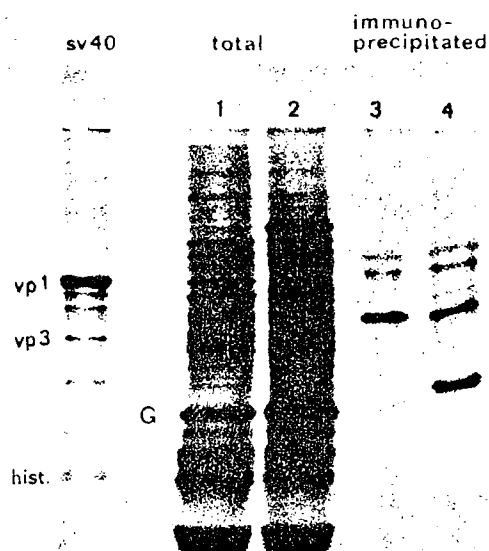


Fig. 2. Electrophoresis of total and immunoprecipitated translation products. A 20- μ l reaction containing $8-9 \times 10^5$ counts/min of [35 S]methionine-labeled translation products obtained with 0.1 μ g polyadenylated mRNA from either poly(rI)·poly(rC)-induced FS11 cells (lanes 2 and 4) or non-induced cells (lanes 1 and 3) was analyzed. For immunoprecipitation (lanes 3 and 4), 20 μ l of interferon antiserum was added, followed 1 h later by 40 μ l of a 50% protein-A—Sepharose CL4B suspension. After 1 h the Sepharose pellet was washed in NaCl/P_i, the proteins were solubilized in electrophoresis sample buffer (containing dodecylsulfate), heated to 100°C for 5 min and loaded on a 12% polyacrylamide gel in dodecylsulfate. For total products (lanes 1 and 2), the reaction was heated in dodecylsulfate sample buffer and loaded directly on the polyacrylamide gel in dodecylsulfate. [35 S]Methionine-labeled SV40 protein markers were run (M_r for VP1 = 46000, VP3 = 29000, histones = 13000–14000). The position of globin (G) is indicated. The gel was fluorographed and exposed 48 h. Arrow shows the 23000- M_r band

polypeptide has a strong tendency to stick to the walls of glass and plastic tubes. If at the end of the translation the reaction medium is removed from the tube, about 10% of the [35 S]methionine incorporated into hot-acid-insoluble material remains on the walls of the tube. Washing the tube with buffer solutions of various ionic strengths does not remove this bound radioactivity. The adsorption phenomenon is not only hydrophobic, since 50% ethyleneglycol did not prevent it. Use of dodecylsulfate, on the other hand, allows the solution of the tube-wall-bound proteins: Fig. 6 (lane I) shows that this material is enriched in the 23000- M_r polypeptide. This band is absent when mRNA from non-induced FS11 cells is used (Fig. 6, lane NI). About half of the 23000- M_r band present at the end of the translation reaction can in this way be recovered from the tube walls. This observation has several consequences. First, if care is not taken

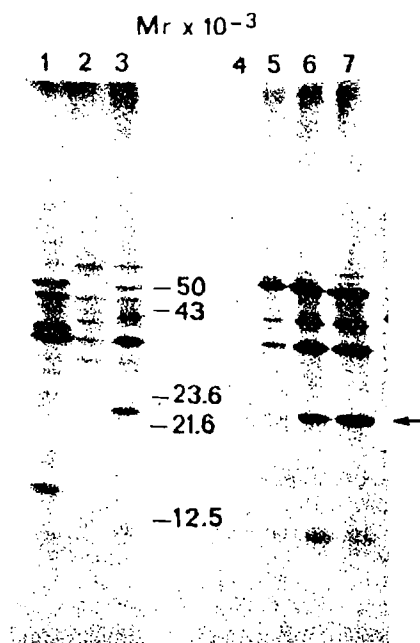


Fig. 3. Specificity of immunoprecipitation. A 25- μ l reaction containing 350000 counts/min of [35 S]methionine-labeled translation products obtained with 0.16 μ g polyadenylated mRNA from poly(rI)·poly(rC)-induced FS11 cells was immunoprecipitated with 25 μ l interferon antiserum (lane 3) or 25 μ l non-immune serum (lane 2). In lane 1, a translation reaction with 0.5 μ g polyadenylated mRNA from non-induced FS11 (440000 counts/min of [35 S]methionine) was immunoprecipitated with interferon antiserum. Lanes 4–7 were like lane 3, but interferon antiserum was diluted 1:1250 (lane 4), 1:250 (lane 5), 1:50 (lane 6) or 1:10 (lane 7) with NaCl/P_i before use. After 1 h, 12.5 μ l of a 10% *Staphylococcus* suspension was added to each mixture and the precipitate transferred to another tube and washed with NaCl/P_i and processed for electrophoresis on a 10–20% polyacrylamide gel in dodecylsulfate as in Methods. IgG heavy chain, ovalbumin, α -chymotrypsinogen, α -chymotrypsin and cytochrome c were used as markers to calibrate the gel. Arrow shows the 23000- M_r band

to use a large volume with respect to the tube surface and to avoid strong agitation, much of this product may be lost in the tube. Second, the immunoprecipitated material has to be transferred to a clean tube before the dodecylsulfate-containing electrophoresis sample buffer is added (see Methods); otherwise, tube-bound material may contaminate the immunoprecipitate and elevate the background of the non-immune serum precipitate. Thirdly, the wall-sticking may be used to detect quickly the 23000- M_r polypeptide among the translation products.

As an example, Fig. 6 shows the analysis by sucrose gradient of the mRNA coding for the 23000- M_r polypeptide. Polyadenylated mRNA from induced FS11 cultures was heated in formamide and sedimented through a sucrose gradient. Each fraction was precipitated with ethanol and translated in reticulocyte lysates. The tubes in which translation was carried out

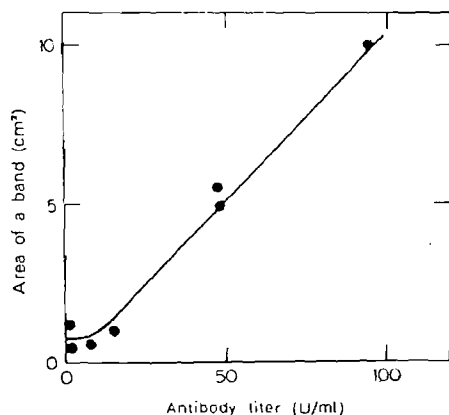


Fig. 4. Correlation between immunoprecipitation and anti-interferon titer. A 5- μ l aliquot from a translation reaction with mRNA from induced cells were immunoprecipitated (as in Fig. 3) with 5 μ l sera from four different rabbits taken either before immunization, or at different times during immunization with interferon. The anti-interferon titer of each serum was determined as described in Methods. The area of the 23000- M_r polypeptide band was measured after gel electrophoresis of each immunoprecipitate by scanning the autoradiograph.

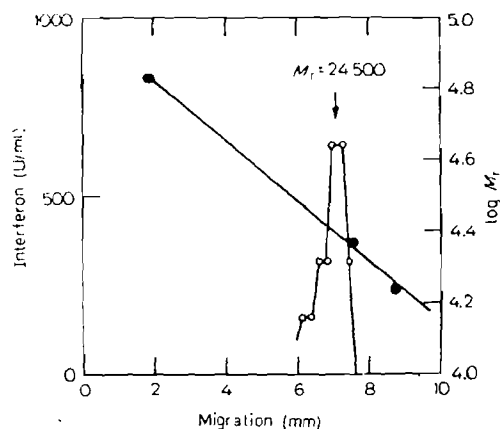


Fig. 5. Polyacrylamide gel electrophoresis in dodecylsulfate of human fibroblast interferon. 10^4 U of interferon produced by FS11 cells and partially purified by chromatography on CM-Sephadex (see Methods) was loaded on a cylindrical 10% polyacrylamide gel in dodecylsulfate. Electrophoresis was for 2 h at 4 mA/gel. Slices of 2 mm were extracted with 0.2 ml NaCl/ P_{10} , 1 h at room temperature, and diluted in medium with 50% fetal calf serum. The inhibition of VSV cytopathic effect was used to determine the antiviral titer. Molecular weight markers were run on a separate gel and stained with Coomassie blue.

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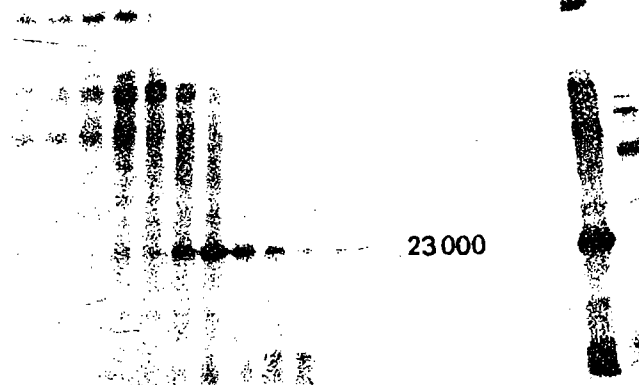


Fig. 6. Sucrose gradient analysis of interferon mRNA. Polyadenylated mRNA from poly(rI)-poly(rC)-induced FS11 cells was dissolved in 37.5% formamide (Fluka), 0.1% sodium dodecylsulfate, 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl pH 7.5 and heated to 40°C for 25 min. About 10 μ g of RNA were layered on a 15–30% sucrose gradient in 0.5% sodium dodecylsulfate, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5 and centrifuged for 16 h in a Spinco rotor SW60 at 20°C. A total of 19 fractions were collected. 5 μ g tRNA was added to each fraction prior to precipitation with 2 vol. ethanol in 0.2 M sodium acetate pH 5. The RNA was washed in ethanol, dried, dissolved in 20 μ l water; 2 μ l were used for translation in 25- μ l protein-synthesis reactions as in Methods. The tubes were strongly agitated and the tube-wall-bound, [35 S]methionine-labeled translation products dissolved in electrophoresis sample buffer and loaded on a 12% polyacrylamide gel in dodecylsulfate. The 23000- M_r polypeptide, which comigrated with that immunoprecipitated by interferon antiserum, was seen in the translation products of total induced mRNA (lane I) but not in those of total non-induced mRNA (lane N1). Without mRNA (lane O) there was no tube-bound radioactive proteins. The products of the RNA from sucrose gradient fractions 1 (bottom) to 14 only are shown. *E. coli* RNA markers run in parallel showed that 23-S RNA sedimented to tubes 5–6, 16-S RNA to tube 8 and 4-S RNA to tube 18. The mRNA coding for the 23000- M_r polypeptide sediments as a 14-S RNA species (sucrose gradient fraction 9).

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were emptied and electrophoresis buffer with dodecyl-sulfate introduced. The results of the polyacrylamide gel electrophoresis are shown in Fig. 6 (lanes 1–14). The 23000- M_r polypeptide, which was seen only with mRNA from induced cells, is translated from an mRNA which sediments at 14 S (\approx 950 nucleotides). Identical results were obtained when the immunoprecipitation technique was used.

Immunoprecipitation or tube-wall attachment were used to follow the 23000- M_r polypeptide synthesis under various conditions. We found that 70 mM potassium acetate and 0.25–0.3 mM spermidine give optimum translation. Maximum synthesis was seen after 60 min of incubation. When mRNAs were prepared at different times after induction of FS11 cells with poly(rI) · poly(rC) and cycloheximide, it was found that the mRNA activity for the synthesis of the 23000- M_r polypeptide appears maximum at 3.5–6.5 h, while it is much lower at 2 h and absent when cells are extracted immediately after addition of the inducers.

DISCUSSION

Immunoprecipitation and polyacrylamide gel electrophoresis of the cell-free translation products of mRNA from human fibroblasts, in which interferon formation has been induced, demonstrates the synthesis of a specific polypeptide of M_r 23000. No other cell-free product fits the criteria defining an interferon mRNA product, such as (a) requirement for induction of the cells by poly(rI) · poly(rC), (b) specific immunoprecipitation by interferon antiserum and (c) molecular size. When compared by polyacrylamide gel electrophoresis in dodecylsulfate, the mobility of the immunoprecipitated polypeptide was very close to the mobility of interferon's biological activity. Interferon is known to be a glycoprotein [23–25] and it is questionable whether the polypeptide made *in vitro* in reticulocyte lysates is glycosylated. Carbohydrates with M_r of several thousand are attached to the interferon polypeptide and their removal reduces significantly the size of the interferon molecule [25]. If it lacks these carbohydrates, the product *in vitro* could, therefore, be smaller than mature interferon. On the other hand, since interferon is an export protein [26], it may be synthesized as a precursor protein with a slightly larger polypeptide size than the mature chain [27,28]. The good agreement between the size of the cell-free product *in vitro* and interferon *in vivo* may be then rather fortuitous. Nevertheless, the evidence presented here supports the conclusion that the 23000- M_r polypeptide is the translation product of human fibroblast interferon mRNA.

Synthesis from mRNA *in vitro* of biologically active, species-specific interferon, with proper anti-

genicity, has been demonstrated by several groups before [1–14]. The aim of this work was to identify the native interferon polypeptide chain, synthesized in a cell-free system. Reynolds et al. [3] have shown that a 25000- M_r polypeptide is made by frog oocytes in response to human fibroblast interferon mRNA. The technique reported here allows a more direct isolation of the translation product and presents several advantages. Since translation in reticulocyte lysates yields products of high specific radioactivity, automatic sequencing of the protein becomes possible. Furthermore, purification of the mRNA should be greatly facilitated by the possibility of following directly the cell-free translation products.

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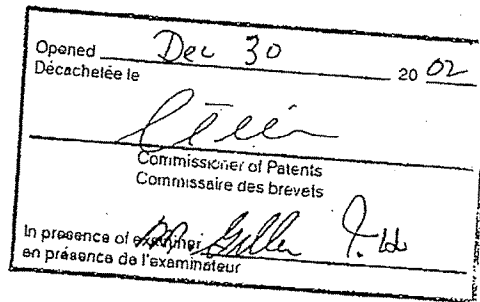
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The nucleotide sequence of a cloned human leukocyte
interferon cDNA.

(Amino acid sequence; restriction map; signal sequence;
interferon synthesis)

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This is EXHIBIT HALEY- 2
to
the Affidavit of James F. Haley, Jr.
sworn before me
this 21st day of November, 2001

Commissioner for Oath or Notary Public

MARIA E. MARLEY
Notary Public, State Of New York
No. 01MA4890484
Qualified in New York County
Commission Expires April 27, 2003

Sugano Exhibit 1008
Fiers v. Sugano
Interference No. 105,661

Sugano EXHIBIT 2047
Sugano v. Goeddel
Interference No. 105,334 and 105,337

SUMMARY

We have determined the nucleotide sequence of the human leukocyte interferon cDNA-containing hybrid plasmid Z-pBR322(Pst)/HcIF-2h, which has been shown to direct the formation of a polypeptide with human leukocyte interferon activity (Nagata et al., Nature, 1980, in press). The 910 base pair insert contains a 567 (or 543) base pair coding sequence, which determines a putative preinterferon polypeptide consisting of a signal peptide of 23 (or less likely 15) amino acids, followed by an interferon polypeptide of 166 amino acids (calculated molecular weight, 19'390). The coding sequence is preceded by a (most likely incomplete) 56 bp leader and followed by a 242 bp trailer and 7 A residues from the poly(A) tail. A comparison of the sequence of 35 amino terminal amino acids of lymphoblastoid interferon (Zoon et al., Science 207, 527-528, 1980; M. Hunkapiller and L. Hood, personal communication) and the corresponding sequence deduced for leukocyte interferon revealed 9 differences. This suggests that these two interferons are encoded by two non-allelic genes.

INTRODUCTION

We have recently described the isolation of a hybrid plasmid Z-pBR322(Pst)/HcIF-2h, or Hif-2h for short, which contains a cDNA sequence coding for human leukocyte interferon. The hybrid DNA was identified by its capacity to (a) hybridize with human leukocyte interferon mRNA and (b) to direct the synthesis in E.coli of a protein with properties of human leukocyte interferon (Nagata et al., 1980).

In this paper we report the nucleotide sequence of the 910 bp insert of Hif-2h. Two AUG triplets and a UAA termination codon, all in the same reading frame, define a stretch of 567 or 543 nucleotides which encodes a polypeptide of 166 amino acids corresponding to the interferon polypeptide proper, preceded by 23 or 15 amino acids, which may constitute a signal sequence. The coding region is flanked at the 5' end by 79 nucleotides, 23 of which are terminal G residues, and at the 3' end by 264 nucleotides, 15 of which are terminal C residues.

MATERIALS AND METHODS

Plasmid DNA was prepared by method B described in Wilkie et al. (1979). EcoRI was a gift from W. Boll and BspI from A. Kiss. All other restriction enzymes were purchased from New England Biolabs and used in essence as recommended by the supplier (except that 200 µg/ml gelatin replaced bovine serum albumen in the enzyme buffers). Carrier-free [γ -³²P]ATP was prepared by an unpublished procedure of B. Seed.

5'-terminal labeling of DNA.

Restricted DNA (20 μ g) was extracted with phenol, precipitated with ethanol, dissolved in 0.05 M Tris-HCl (pH 8), and passed over a small column of Chelex-100. Fragments with flush or 5'-overhanging ends were dephosphorylated by treatment with 0.2 units calf intestinal alkaline phosphatase (Boehringer) per pmol DNA 5' ends in 200 μ l 0.05 M Tris-HCl (pH 8) for 60 min at 37°C. The enzyme was inactivated by heating 60 min at 65°C. For DNA fragments with 3' overhanging ends, bacterial alkaline phosphatase (Worthington) was used as described (Maxam and Gilbert, 1977), except that incubation was at 65°C for 30 min. The dephosphorylated DNA was purified by adsorption to and elution from DEAE-cellulose as described (Müller et al., 1978) or subjected to polyacrylamide gel electrophoresis where required (see below). Fragments recovered from a polyacrylamide (or agarose) gel in 0.15 M NaCl, 0.05 M Tris-HCl (pH 8) were adsorbed to a 0.1-ml hydroxyapatite (Biorad HTP) column, washed with 4 times 1 ml of 0.1 M potassium phosphate buffer (pH 7) and eluted with 0.3 ml 1 M potassium phosphate buffer (pH 7). The solution was diluted tenfold and the DNA adsorbed to DEAE cellulose and recovered as described (Müller et al., 1978).

After ethanol precipitation, the DNA was 5'-terminally labeled with [γ -³²P]ATP (12-34 μ Ci per pmol DNA end) and polynucleotide kinase (New England Biolabs or P-L Biochemicals Inc.) essentially as described (Maxam and Gilbert, 1977), except that the DNA

was not denatured before the kinase reaction. Specific activities of 1-1.5 μCi [^{32}P]phosphate per pmol DNA 5'-ends were obtained.

Nucleotide sequence determination.

For sequencing, labeled fragments were cleaved with a second restriction enzyme and the products separated by electrophoresis through a 5% polyacrylamide gel in Tris-borate-EDTA buffer. The desired fragments were extracted from the gel and purified as described (Müller et al., 1978). The various fragments for sequencing were prepared as follows (the number indicates the nominal fragment chain length in base pairs, the labeled site is indicated by an asterisk, and the letters in parentheses refer to the arrows shown in Fig. 1): (a) and (b), cleavage of Hif-2h with BspI, isolation by 5% polyacrylamide gel electrophoresis in Loening's buffer (Loening, 1967) of Bsp-Bsp-232 (for (a)) and Bsp-Bsp-949 (for (b)), labeling, cleavage with PstI, isolation of (a) Bsp*-Pst-83 and (b) Bsp*-Pst-827. (c) and (d), cleavage of Hif-2h with BglII, labeling, cleavage with PstI, isolation of (c) Bgl*-Pst-336 and (d) Bgl*-Pst-570. (e) and (f), cleavage of Hif-2h with MboII, labeling, digestion with PstI and HindII (to cleave an interfering 350 bp pBR322 fragment), isolation of (e) Mbo*-Pst-519 and (f) Mbo*-Pst-351. (g) and (h), cleavage of Hif-2h with EcoRI, labeling, cleavage with PstI, isolation of (g) Eco*-Pst-708 and (h) Eco*-Pst-198. (i) and (j), cleavage of Hif-2h with PstI, labeling, cleavage with BglII, isolation of (i) Pst*-Bgl-570 and (j) Pst*-Bgl-336. (k) and (l), cleavage of Hif-2h with AvaII, labeling, cleavage with PstI and BglII, isolation of (k) Ava*-Pst-186 and (l)

Ava⁺-Bgl-147. (m) Cleavage of plasmid with PvuII, labeling, cleavage with PstI and BglII, isolation of Pvu⁺-Pst-486. The fragments were degraded according to Maxam and Gilbert (1977), with the modifications described in protocols provided by the same authors in September, 1978. The products were fractionated on 0.1 x 25 x 36 cm 12% polyacrylamide gels (acrylamide/bis-acrylamide = 18/1) in 50 mM Tris-borate, 1 mM EDTA (pH 8.3), with runs of 2, 8, 18 and 26 h at 900 V following a 6 h prerun at 700 V. Best results were obtained when the gels were kept at room temperature 2-3 days before use.

RESULTS

1) Physical map of Hif-2h DNA.

Hif-2h consists of dC-elongated human Le IF cDNA joined to pBR322 (Bolivar et al., 1977) which had been cleaved with PstI and elongated with dG residues. A physical map was prepared by measuring the lengths of the fragments generated by single cleavage with EcoRI, BspI, PstI and MboII and double cleavages with PstI on the one hand and EcoRI, BglII, BspI and MboII on the other, as well as with EcoRI and BglII, and EcoRI and MboII. In addition, DNA fragments which were ³²P-labeled at one 5' end, were partially digested with a variety of restriction enzymes, and the lengths of the labeled products determined (Smith and Birnstiel, 1976). The resulting preliminary map was used as a basis for the nucleotide sequence analysis; the map shown in

Fig. 1 was refined (cf. also Fig. 3) using the results of the nucleotide sequence analysis described below. No restriction targets for BglI, KpnI, HaeII, XhoI, PvuI, XbaI, PstI, BstEII, BamHI, HindII, SalI, HindIII, HpaII, TaqI, HgaI, Taci, HpaI or HhaI were found in the insert; there were single sites for BspI, BglII and EcoRI, and two sites for PvuII. One each of 4 AvaII and 4 MboII targets (marked with * in Fig. 3) was not cleavable by the cognate enzyme, perhaps because of methylated bases in adjacent EcoRII and MboI sites. The MboI sites were not cleavable.

2) The orientation of the coding sequence.

In order to determine the orientation of the coding strand relative to pBR322 the experiment outlined in Fig. 2a was carried out. The hybrid plasmid Hif-2h was cleaved at the single BglII site, 5'-terminally labeled with [^{32}P] phosphate and digested with PstI to yield 336/344 and 578/570 bp radioactive fragments. The fragments were denatured, annealed with poly(A) RNA from induced leukocytes, and the mixture was treated with S_1 nuclease. The resulting products were denatured and analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 2b, a ^{32}P -labeled fragment of about 340 nucleotides was protected. In a second, similar experiment the labeled fragments were first separated and then annealed individually with poly(A) RNA: the shorter, but not the longer probe was protected against S_1 nuclease (data not shown). These experiments identify the 5' labeled 344 nucleotide strand as the minus strand, i.e. the strand complementary

to the mRNA. Therefore, the orientation of the insert is such that the coding strand of the IF cDNA is a continuation of the coding strand of the β -lactamase (Amp) gene, as shown in Fig. 2a. Fig. 2b also shows that poly(A) RNA from non-induced leukocytes, added to the hybridization at a similar level as induced poly(A) RNA, did not protect the labeled IF cDNA probe.

3) Nucleotide sequence analysis.

Hif-2h DNA was cleaved by an appropriate restriction enzyme, labeled with [32 P]phosphate at the 5' termini, and digested with a second restriction enzyme to yield fragments labeled at only one 5' end; the isolated fragments were sequenced by the Maxam-Gilbert procedure (Maxam and Gilbert, 1977). Fig. 1 shows the fragments analyzed in this fashion. Each stretch of the cDNA insert was sequenced from both strands, and each restriction site which served as labeled terminus was sequenced using a fragment spanning it. The nucleotide sequence thus obtained is shown in Fig. 3. The heteropolymeric part of the insert is flanked by 23 G residues at the 5' end and by 7 A residues (probably reflecting the poly(A) terminus of the mRNA) followed by 15 C residues at the 3' terminus. An AUG initiation triplet in position 57-59 and a UAA termination triplet at position 624-626 define a reading frame uninterrupted by nonsense codons. Both other reading frames contain 18 and 12 nonsense codons, respectively. The only other sequences flanked by an AUG

(or GUG) and by a termination triplet, which could code for a polypeptide of 25 amino acids or more, lie in different reading frames, between nucleotides 226 and 304, 640 and 778, and 683 and 743, respectively.

Hood and his colleagues have recently determined the sequence of 35 amino terminal amino acids of human lymphoblastoid interferon (Zoon et al., 1980; M. Hunkapiller and L. Hood, personal communication). In Fig. 5 the sequence of human lymphoblastoid IF (B) is aligned with the amino acid sequence determined by the major reading frame of the Hif-2h nucleotide sequence (A) such that the amino terminal amino acid of the former coincides with the amino acid coded for by the 24th codon of the latter. Extensive coincidence is found: 26 of 35 positions have identical amino acids. This confirms the assignment of the reading frame.

DISCUSSION

Cloned cDNA generated from poly(A) RNA by commonly used procedures (Efstratiadis et al., 1977) lacks 5' terminal nucleotides and may even contain artifactual sequences (Richards et al., 1979). It is therefore not certain whether the first AUG of the cloned human Le IF cDNA Hif-2h, which is located 57 nucleotides downstream from the 5' terminus of the heteropolymeric sequence, in fact corresponds to the first AUG on the mRNA.

Bearing these reservations in mind, we shall assume, until further experimental evidence becomes available, that this is the case.

In eukaryotic mRNAs the first AUG triplet from the 5' terminus is usually the initiation site for protein synthesis (Kozak, 1978). The codon in the cloned human Le IF cDNA corresponding to the first amino acid of lymphoblastoid interferon is 22 codons downstream from the first AUG (and 14 codons downstream from the second one) indicating that the sequence coding for interferon may be preceded by a sequence determining a signal peptide of 23 (or less likely 15) amino acids. The longer of the presumptive signal sequences contains an uninterrupted series of 11 hydrophobic amino acids (and the shorter one, one of 6). This accumulation of hydrophobic residues is characteristic of signal sequences (cf. Davis and Tai, 1980). The presumptive cleavage site between signal and interferon sequence lies between a Gly and a Cys residue. It is striking that in the case of E.coli prelipoprotein, cleavage occurs between the same two amino acids (Inouye et al., 1977). It will be interesting to determine whether the postulated preinterferon exists, and if so, whether it is correctly processed in E.coli, especially in view of our finding (S. Nagata, unpublished results) that about 50% of the interferon activity produced in E.coli can be released by osmotic shock and is therefore located in the periplasmic space (Anraku, 1968).

The sequence corresponding to (mature) Le IF polypeptide comprises 498 nucleotides, which code for 166 amino acids. Assuming that there is no carboxyterminal processing, the molecular weight of the interferon polypeptide, as calculated from Table 1, is 19'388. The base composition of the coding sequence is 50% GC; the codon usage within the interferon coding sequence (Table 2) is in reasonable agreement with that compiled for mammalian mRNAs in general (Grantham et al., 1980); the deviations observed may be ascribed to the small numbers involved.

The 3' non-coding region consists of 242 nucleotides; this length is intermediate between that of chicken ovalbumin mRNA (637 residues) (McReynolds et al., 1978) and rat insulin mRNA (53) (Ullrich et al., 1977). The high AT content (69%) is similar to that found for the corresponding segment of mouse β -globin minor mRNA (63%) (Konkel et al., 1979); the AT content of eukaryotic 3' non-coding regions range from 94% in mRNA yeast mitochondrial ATPase (Hensgens et al., 1979) to 42% in bovine ACTH- β LPH mRNA (Nakanishi et al., 1979). No striking homologies to 3' non-coding regions of other mRNAs were noted, except for the AATAAA(AC) sequence 18-27 nucleotides upstream from the poly(A) sequence, found previously (Proudfoot and Brownlee, 1976) in almost all eukaryotic mRNAs examined, at about the same relative position.

The comparison of the first 35 amino acids of lymphoblastoid interferon (Zoon et al., 1980; M. Hunkapiller and L. Hood, personal communication) and the sequence deduced from Hif-2h (Fig. 5) shows 9 differences. In all cases, the codons for the differing amino acids could be related by one-base changes. The amino acid compositions (Table 1) determined directly for lymphoblastoid interferon on the one hand and deduced from the Hif-2h sequence on the other, show striking differences in regard to their content of Gly, Pro, Cys and Met. These differences are too large to be explained by polymorphism; most likely we are dealing with the products of two non-allelic genes, since the degree of divergence of the two proteins (26% mismatch) is similar to that between, for example, human and sheep β globin (23% mismatch). We have recently surveyed our human leukocyte cDNA clone bank and identified a hybrid plasmid (Z-pBR322(Pst)HcIF-II-206, or Hif-II206 for short) which also directs synthesis of interferon activity in *E. coli* and has a different restriction pattern than Hif-2h (M. Streuli and M. Schwarzstein, unpublished results). This clone represents a second leukocyte interferon gene (Le-IF II), differing from the one (Le-IF I) which corresponds to Hif-2h. The amino acid composition of an IF preparation from human leukocytes (Rubinstein et al., 1979) agrees somewhat better than that of lymphoblastoid IF with the amino acid composition deduced for Le-IF I (Table 1).

Taniguchi and his colleagues prepared cDNA from induced fibroblast poly(A) RNA and selected presumptive interferon cDNA clones by hybridization techniques (Taniguchi et al., 1979). The nucleotide sequence of one such clone was determined and could be correlated (Taniguchi et al., 1980) with the sequence of the 13 amino terminal amino acids of fibroblast

interféron (Knight et al., 1980). The striking structural homologies between the leukocyte and fibroblast interferon cDNA sequences will be analyzed elsewhere.

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TABLE 1 Amino acid composition of leukocyte and lymphoblastoid interferon.^{a)}

	Leukocyte IF, deduced from nucleotide sequence of Hif-2h cDNAb)	Lymphoblastoid IF ^{c)}	Leukocyte IFd)
Asn	6	} 15	15
Asp	11		
Thr	9	8	7.5
Ser	13	11	8
Gln	10	} 27	24
Glu	15		
<u>Pro</u>	<u>6</u>	<u>11</u>	6
<u>Gly</u>	<u>3</u>	<u>11</u>	5.5
Ala	10	11	8
<u>Cys</u>	<u>5</u>	<u>2</u>	3
Val	6	8	8
<u>Met</u>	<u>6</u>	<u>3*</u>	-
Ile	7	7	9
Leu	22	18	22
Tyr	4	4	5
Phe	8	7	9
His	3	4	3
Lys	8	10	12
Arg	12	10	7
Trp	2	1	1

TABLE 1 (cont.)

- a) Values believed to differ significantly are underlined.
- b) From Fig. 3.
- c) From Zoon et al. (1980), except for the value marked with *, which was from L. Hood (personal communication).
- d) From Rubinstein et al. (1979).

TABLE 2 Codon usage in the interferon coding sequence^{a)}.

Codon		all MAM	IG	MAM-IG	Le IF
Arg	CGA	2	7	0	6
	CGC	9	4	12	0
	CGG	6	3	8	0
	CGU	7	1	9	0
	AGA	6	12	3	36
	AGG	11	9	11	30
Leu	CUA	8	11	7	6
	CUC	24	26	24	48
	CUG	51	16	68	42
	CUU	7	7	7	0
	UUA	4	11	1	12
	UUG	7	10	6	24
Ser	UCA	11	24	5	12
	UCC	20	17	22	24
	UCG	4	1	5	0
	UCU	17	29	11	24
	AGC	21	27	18	18
	AGU	17	33	9	0
Thr	ACA	13	26	7	18
	ACC	25	30	23	24
	ACG	7	4	9	0
	ACU	19	36	11	12

TABLE 2 (cont.)

Pro	CCA	12	20	7	6
	CCC	18	13	21	12
	CCG	8	5	9	0
	CCU	13	13	12	18
Ala	GCA	11	20	6	12
	GCC	34	22	40	18
	GCG	5	1	8	6
	GCU	23	21	24	24
Gly	GGA	10	20	5	12
	GGC	28	16	34	6
	GGG	11	9	11	0
	GGU	18	25	15	0
Val	GUA	3	5	1	0
	GUC	16	23	13	12
	GUG	35	17	44	18
	GUU	7	7	7	6
Lys	AAA	15	17	14	24
	AAG	45	24	56	24
Asn	AAC	29	29	29	24
	AAU	10	13	9	12
Gln	CAA	10	11	9	12
	CAG	32	30	32	48
His	CAC	22	8	29	6
	CAU	10	11	10	12

TABLE 2 (cont.)

Glu	GAA	22	21	23	36
	GAG	36	23	43	54
Asp	GAC	27	22	30	36
	GAU	18	23	16	30
Tyr	UAC	20	20	20	18
	UAU	15	17	14	6
Cys	UGC	12	6	16	6
	UGU	11	18	8	24
Phe	UUC	33	30	35	24
	UUU	16	13	18	24
Ile	AUA	4	7	3	0
	AUC	20	21	19	42
	AUU	11	18	8	0
Met	AUG	16	14	16	36
Trp	UGG	16	25	12	12

a) The values are expressed per 1000 amino acid residues.

The data for "all MAM" (compiled from 18 mammalian sequences), "IG" (6 immunoglobulin sequences) and "MAM-IG" (12 non-immunoglobulin mammalian sequences) are from Grantham et al. (1980), those for human leukocyte interferon from Fig. 3.

FIGURE LEGENDS

Fig. 1 Strategy for the determination of the nucleotide sequence of Hif-2h DNA.

The restriction map was determined as outlined in the text and subsequently refined using the results of the nucleotide sequence analyses shown in Fig. 3. The filled circles represent labeled 5' termini, the solid arrows indicate the sequences read off the labeled fragments. The dashed lines represent regions not read off a particular fragment. Black box, interferon coding sequences; hatched box, putative signal sequence; white box, non-coding region. Straight lines, homopolymeric dG:dC flanking regions; wavy line, pBR322.

Fig. 2 Determination of the orientation of the Le IF coding sequence.

(a) An outline of the approach. Hif-2h DNA is cleaved asymmetrically within the IF cDNA sequence, at the BglII site. The 5' termini are labeled with [32 P] (filled circles) and the DNA cleaved with PstI. The labeled fragments, either separated or not, are denatured, hybridized with poly(A) RNA from IF-producing leukocytes, and the mixture digested with S_1 nuclease. If the coding sequence has the orientation

shown in the figure (as we found to be the case), the smaller (344 nucleotide) BglII fragment is recovered; if the orientation had been the opposite, the larger (578 nucleotide) BglII fragment would have been protected by the mRNA. +, sense strand; -, antisense strand; Amp, ampicillinase gene; Tet, tetracycline gene. The arrows indicate the direction of transcription. PN, polynucleotide kinase.

(b) Hif-2h DNA was cleaved, labeled and recleaved as outlined above. The specific ^{32}P -radioactivity was 1.3×10^6 cpm/pmol end. 0.015 pmol of probe in 5 μl hybridization buffer (80% formamide, 40 mM PIPES (pH 6.4), 0.4 M NaCl, 0.001 M EDTA) were denatured for 10 min at 60°C and transferred to a tube in which 0.15-5 μg Le poly(A) RNA had been dried down. In other similar experiments 25 μg oligo(C) were added to prevent protection of the dC residues of the probe by the dG residues of the probe or the DNA strand complementary to it. The mixture was heated 19 h at 48°C in a sealed capillary and transferred to 0.1 ml S_1 buffer (250 mM NaCl, 30 mM NaAc buffer (pH 4.5), 1 mM ZnSO_4) containing 1.5 μg denatured salmon sperm DNA and 55 units S_1 nuclease (prepared according to Wiegand et al. (1975) by A. Schamböck) were added. After 40 min at 30°C each sample was extracted with phenol-chloroform, 10 μg yeast

RNA were added and the nucleic acid was precipitated with 2 vol ethanol. The precipitate was dissolved in 5 μ l 90% formamide, 2 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and analyzed by electrophoresis through a 5% polyacrylamide gel as described (Weaver and Weissmann, 1979). Autoradiography with an Ilford intensifying screen was for 18 h at -70°C . Lane 1, pBR322 cleaved with BspI and 5'-terminally labeled as marker (Sutcliffe, 1978); lane 2, the untreated, labeled probe; lanes 3-5, the labeled probe hybridized with 0.5, 1.5 and 5 μ g uninduced poly(A) RNA, respectively; lanes 6-8, the labeled probe hybridized to 0.5, 1.5 and 5 μ g induced poly(A) RNA, respectively.

Fig. 3

The nucleotide sequence of Hif-2h IF cDNA.

The nucleotide sequence was determined as indicated in the Methods section. The amino acid sequence was deduced from the nucleotide sequence; lower case letters indicate the putative signal polypeptide. The MboI sites as well as the restriction targets marked with * were not cleavable.

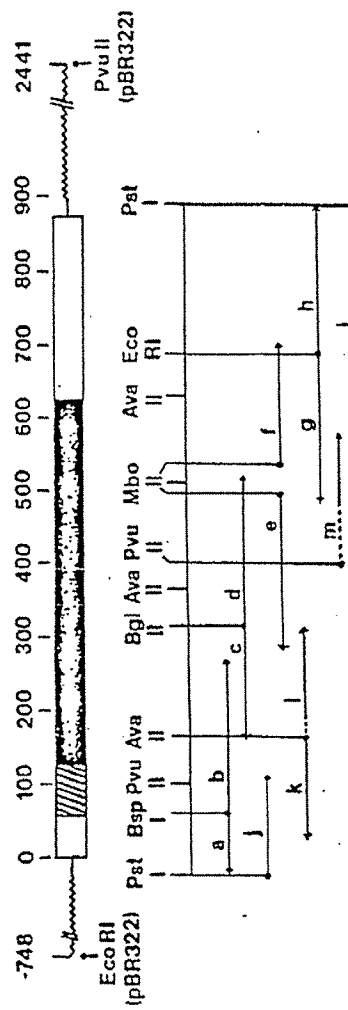
Fig. 4 Autoradiogram of the sequence gel showing the presumed signal polypeptide coding region and the beginning of the interferon polypeptide.

Fragment b (Fig. 1) was degraded as described in the Methods section and analyzed on a 12% gel. The runs were for 2, 8, 18 and 26 h at 900 V. The four lanes for each run show, from left to right, degradations specific for G, A+G, C+T and C. Because of methylation at EcoRII sites, the Cs marked with asterisks are present as gaps in this sequence. Their existence was confirmed by analyses of the other strand.

Fig. 5 Comparison of the amino terminal amino acid sequence of lymphoblastoid interferon (determined experimentally) and leukocyte interferon (deduced from the Hif-2h cDNA nucleotide sequence).

The leukocyte interferon sequence (A) is from Fig. 3; the lymphoblastoid interferon sequence (B) is from Zoon et al. (1980), and M. Hunkapiller and L. Hood (personal communication). Dashes indicate identical amino acids.

Fig. 1



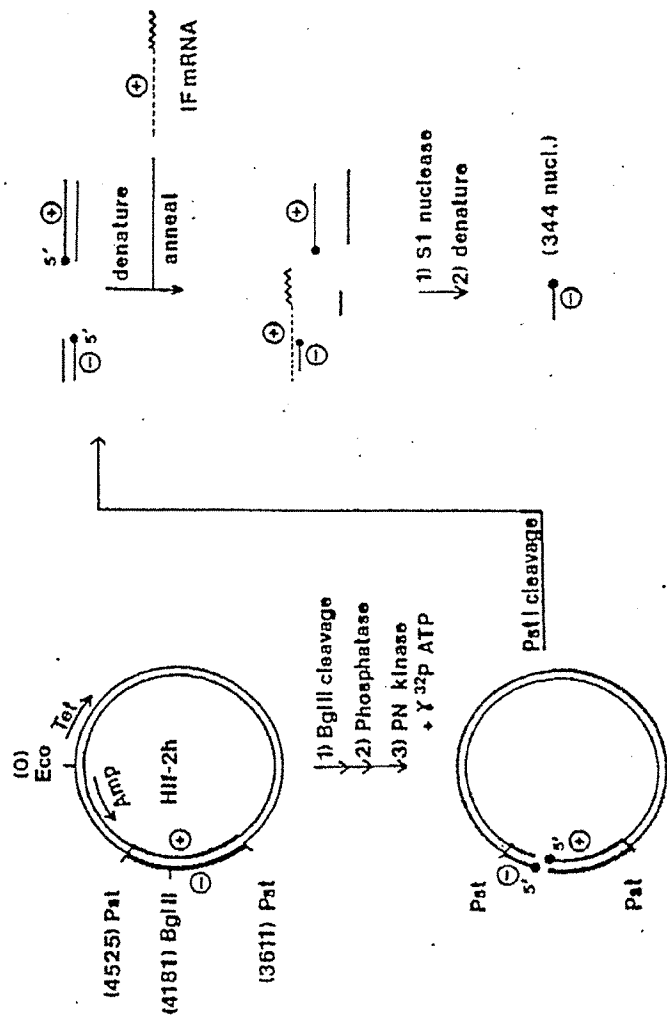


Fig. 2a

a

Fig. 2b

0- 1 2 3 4 5 6 7 8

587 -

434 -

267 -

b.

Fig. 3

[illegible]

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A)	CYS	ASP	LEU	PRO	GLU	THR	HIS	SER	LEU	ASP	ASN	ARG	ARG	THR	LEU
B)	SER	-	-	-	GLN	-	-	-	-	GLY	-	-	-	ALA	-
A)	MET	LEU	LEU	ALA	GLN	MET	SER	ARG	ILE	SER	PRO	SER	SER	CYS	LEU
B)	ILE	-	-	-	-	-	GLY	-	-	-	LEU	PHE	-	-	-
A)	MET	ASP	ARG	HIS	ASP										
B)	LYS	-	-	-	-										

FIG. 5

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DOCUMENT NO: 45

Dec 30 02
[Signature]
M. G. L. J. H.
en présence de l'examinateur

SUGANO EXHIBIT 1009
FIERS V. SUGANO
INTERFERENCE NO. 105,661



This is EXHIBIT FIERS-8
to
the Affidavit of Walter C. Fiers
sworn before me
this 19th day of November, 2001

Commissioner for Oath or Notary Public

after SDS-polyacrylamide gel electrophoresis, the eluate should be centrifuged at 20,000 rev/min (Sorvall SS-34 rotor) for 20 minutes to remove particulate matter before dialysis. Coomassie blue staining of the gels to locate protein bands does not interfere with subsequent sequenator analysis.

New technologies such as the improved amino acid sequencing method described above lead to new research opportunities. With the greater sensitivity provided by this technique, we now can obtain amino acid sequence information on both proteins and peptides with submicrogram (picomole) quantities. This sensitivity should permit analysis of biomedically relevant molecules—such as the interferons—that can only be obtained in microgram quantities, and this ability opens possibilities for further study of these molecules. For example, knowledge of the amino acid sequence permits the synthesis of corresponding DNA probes and opens the possibility of

new strategies for isolating genes, such as those for interferons, that express low levels of messenger RNA's (8).

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9. Supported by a gift from the Ben Weingart Foundation.

29 November 1979

Human Fibroblast Interferon: Amino Acid Analysis and Amino Terminal Amino Acid Sequence

Abstract: The purification of human fibroblast interferon has been simplified to a two-step procedure consisting of affinity chromatography on Blue Sepharose and sodium dodecyl sulfate polyacrylamide gel electrophoresis. A preliminary amino acid composition and the sequence of the 13 amino-terminal residues of homogeneous interferon prepared by this method is reported.

Since the discovery of interferon, its purification and chemical characterization have been primary goals of interferon research. Although their attainment has been slow because of the small quantities of interferon proteins avail-

able, purification to homogeneity has now been achieved with some interferons. However, only microgram quantities have been available for characterization—human fibroblast interferon (1, 2), human lymphoblastoid interferon (3), human leukocyte interferon (4), mouse interferon (5)—and only limited structural information has been acquired (4, 6).

A thorough understanding at the molecular level of the numerous phenomena that are caused by interferon in cells in culture and in animals will not be possible until the elucidation of primary and secondary structures of the interferon proteins is achieved. This structural information will permit (i) comparison of amino acid sequences of interferons from various cell types and animal species, (ii) identification of the polypeptide segments involved in binding to interferon-specific cell-surface receptors, and (iii) chemical synthesis of interferons.

We now report an improved procedure for the purification of human fibroblast interferon that can be used to provide enough protein for structural studies.

Using the automated protein microsequencing technique described in (7), we have determined the sequence of the 13 amino acid residues at the amino terminus of the interferon prepared by this method. We also report a preliminary amino acid composition of the protein.

Human diploid fibroblast cells (FS-4) were cultured and interferon was produced (1). Interferon was assayed by a microtechnique (8) with vesicular stomatitis virus as the challenge virus. Interferon units are given in National Institutes of Health human fibroblast interferon units.

The crude interferon, 10 to 15 liters produced in the absence of serum, was made 1M in NaCl and passed at room temperature through a column (4 by 10 cm) of Blue Sepharose (Pharmacia, Inc.) equilibrated with 0.02M sodium phosphate buffer, pH 7.2, containing 1M NaCl. The interferon was retained whereas more than 95 percent of the total protein passed through the column. The interferon was eluted with a mixture of the column buffer and ethylene glycol (1:1), and each fraction was diluted immediately with 0.5 volume of the buffer (Fig. 1a). Fractions containing interferon activity were pooled, diluted with two volumes of the column buffer, and passed through a small (1 by 6 cm) Blue Sepharose column for concentration. The interferon was eluted as described above (Fig. 1b).

Fractions containing interferon were pooled, dialyzed against 1 mM Tris-HCl,

Table 1. Amino acid composition of human fibroblast interferon.

Amino acid	Composition	
	Mole percent	Residues per 20,000 daltons
Asp	11.3	18.9
Thr	4.8	6.8
Ser	6.2	10.5
Glu	13.9	27.0
Pro	1.6	2.7
Gly*	4.6	7.8
Ala	5.9	10.0
Cys†	1.0	1.7
Val	3.3	6.0
Met	1.7	2.9
Ile	5.3	9.0
Leu	12.0	20.4
Tyr	4.4	7.3
Phe	5.5	9.4
His	2.9	4.9
Lys	6.8	11.6
Arg	6.4	10.9
Trp‡	0.6	1.0

*Includes correction for free glycine present in unhydrolyzed protein. †Determined after performic acid oxidation. ‡Determined after hydrolysis with mercaptoethanesulfonic acid.

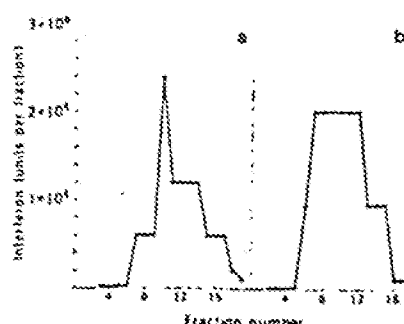


Fig. 1. (a) Fractionation of crude interferon on a large column of Blue Sepharose. Elution of interferon with 50 percent ethylene glycol in column buffer begins at fraction 1. (b) Small Blue Sepharose column. Fractions 7 to 17 in (a) were pooled, passed through the small column, and eluted with 50 percent ethylene glycol in column buffer (fractions 1 to 20).

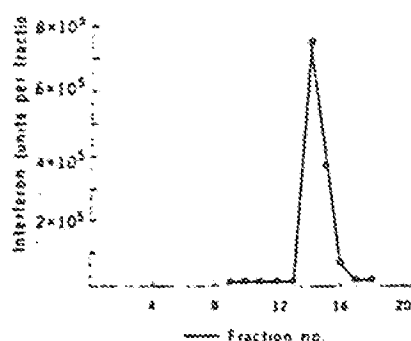


Fig. 2. (a) Preparative electrophoresis of interferon activity profile. Fractions 8 to 13 in Fig. 1b were pooled, concentrated, and subjected to electrophoresis in a polyacrylamide slab gel, 0.75 mm thick. Fractions 14 and 15 were pooled and processed for amino acid sequencing. (b) Polyacrylamide slab gel, staining of proteins eluted from preparative gel in (a). Approximately 2 percent of the protein in fractions 14 and 15 (a) was subjected to electrophoresis and stained. Lanes 1 and 3, standard proteins; lane 2, interferon.

pH 6.8, containing 0.02 percent sodium dodecyl sulfate (SDS, Bio-Rad electrophoresis grade), and concentrated to dryness in a vacuum centrifuge. The interferon was then subjected to electrophoresis on a SDS-polyacrylamide slab gel and eluted (Fig. 2a). Fractions eluted from the gel were assayed for interferon activity (Fig. 2a). Approximately 0.2 μ g of interferon from the peak activity fraction was subjected to electrophoresis in this system again, and the gel was stained with Coomassie blue (Fig. 2b).

The preparative electrophoresis fractions containing interferon were pooled and centrifuged for 30 minutes at 30,000 rev/min at 4°C to remove polyacrylamide gel particles. The interferon solution was dialyzed first against 0.15M NaCl containing 0.1 percent SDS and then against 0.02 percent SDS. The dialyzed interferon was concentrated to dryness in a vacuum centrifuge.

This purification procedure is simpler and shorter than that described previously (1). Recoveries from the large Blue Sepharose column have ranged from 50 to 100 percent, and those from the small column approach 100 percent. The interferon (5×10^5 U/mg) eluted from these columns is stable for at least 4 weeks at 4°C in 1M NaCl, 35 percent ethylene glycol, pH 7.2. Recoveries of activity from the SDS gels have ranged from 5 to 20 percent, and specific activities of this protein have ranged from 2×10^5 to 8×10^5 U/mg. Accurate specific activities are difficult to determine, and

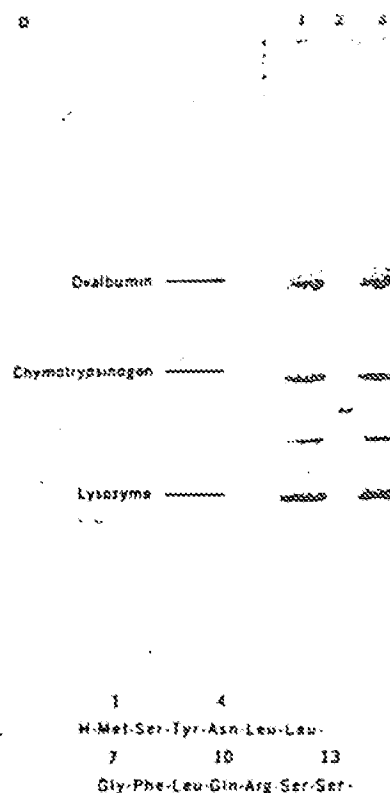


Fig. 3. The amino-terminal amino acid sequence of human fibroblast interferon.

two- to fourfold differences above 1×10^5 U/mg are probably not meaningful. Overall yields of purified interferon from 10- to 15-liter hatches of crude material (5×10^5 to 7×10^5 total units, 8×10^5 U/mg) have averaged around 10 percent. This gives 5 to 10 μ g of homogeneous interferon.

Amino acid analysis on 1- to 2- μ g portions was performed on a Durrum D-500 amino acid analyzer (Table 1). Automated Edman degradation on 0.4- to 2- μ g portions of the purified interferon was performed on a spinning cup sequencer (7). Phenylthiohydantoin (PTH) amino acids were identified by high-performance liquid chromatography (HPLC) on a Du Pont Zorbax CN column (9).

The sequence of the 13 amino terminal amino acid residues of human fibroblast interferon was determined by this microsequencing technique (Fig. 3). Yields of PTH methionine at cycle 1 for three sequencer runs ranged from 60 to 100 percent (based on protein determination by amino acid analysis), and the sequencer repetitive cycle yields were 92 to 95 percent. Any unblocked minor peptide sequence present at > 5 percent of the reported sequence could have been detected by the methods used, but none has

homogeneity of the interferon peptide preparation.

Determining the amino acid sequence of a protein is essential in order to identify its active site and to understand its molecular mechanism of action. Comparison of structural features of interferons from different species and from different cell types within an animal will prove or disprove whether they are different proteins. If there is an active site common to all interferons, it should be identifiable by comparison of the amino acid sequences. Comparison of the amino terminal sequence reported here for human fibroblast interferon does not as yet reveal any apparent homology with the amino-terminal sequence reported for human lymphoblastoid interferon (16). However, there is limited homology (3/13 residues identical) with the 37,000 dalton mouse Ehrlich ascites cell interferon (11).

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30 November 1979

SCIENCE, VOL. 207

25-03-80

Linear DNA

DATE FILED: 05/06/2009
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Acc I sites in INTERFERON

GTAGAC (c2)
0
GTATAC (c2)
0
GTCGAC (c2)
0
GTCTAC (c2)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Opened <u>Dec 30</u> 20 <u>02</u>
Déclarée le <u>Dec 30</u> 20 <u>02</u>
<u>[Signature]</u> Commissioner of Patents Commissaire des brevets
In presence of <u>[Signature]</u> en présence de l'examinateur

Atu BI sites in INTERFERON

CCAGG (c2)
462
CCCGG (c2)
388 430 552

Resulting fragment sizes :
389 42 32 90 297
Tabled according to length :
389 297 90 42 32

Acy I sites in INTERFERON

GGCGCC (c2)
0
GGCGTC (c2)
0
GACGCC (c2)
287
GACGTC (c2)
0

Resulting fragment sizes :
288 562
Tabled according to length :
562 288

SUGANO EXHIBIT 1010
FIERS V. SUGANO
INTERFERENCE NO. 105,661

Asu I sites in INTERFERON

GGGCC
0

(c1)



This is EXHIBIT FIERS-15
to
the Affidavit of Walter C. Fiers
sworn before me
this 19th day of November, 2001

1

Commissioner for Oath or Notary Public

GGACC (cl)
555
GGTCC (cl)
0
GGCCC (cl)
0

Resulting fragment sizes :
555 295
Tabled according to length :
555 295

Ava I sites in INTERFERON

CCCGGG (cl)
0
CTCGGG (cl)
0
CCCGAG (cl)
0
CTCGAG (cl)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Ava II sites in INTERFERON

GGACC (cl)
555
GGTCC (cl)
0

Resulting fragment sizes :
555 295
Tabled according to length :
555 295

Ava III sites in INTERFERON

ATGCAT (cx0)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Avr II sites in INTERFERON

CCTAGG (cx0)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Alu I sites in INTERFERON

AGCT (c2)
118 131 183 264

Resulting fragment sizes :
119 13 52 81 585
Tabled according to length :
585 119 81 52 13

Bam HI sites in INTERFERON

GGATCC (c1)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Bbv I sites in INTERFERON

GCTGC (cx0)
265
GCAGC (cx0)
162 262 268

Resulting fragment sizes :
161 100 3 3 583
Tabled according to length :
583 161 100 3 3

Bcl I sites in INTERFERON

TGATCA (c1)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Bgl II sites in INTERFERON

AGATCT (c1)
629

Resulting fragment sizes :
629 221
Tabled according to length :
629 221

Est cII sites in INTERFERON

GGTGACC (c1)
0
GGTAACC (c1)
0
GGTCACC (c1)
0
GGTTACC (c1)
611

Resulting fragment sizes :
611 239
Tabled according to length :
611 239

Bal I sites in INTERFERON

TGGCCA (c3)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Cla I sites in INTERFERON

ATCGAT (c2)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Cau II sites in INTERFERON

CCGGG (c2)
0
CCCGG (c2)
0

Resulting fragment sizes :
850

Resulting fragment sizes :
850
Tabled according to length :
850

Pvu II sites in INTERFERON

CAGCTG (c3)
263

Resulting fragment sizes :
265 585
Tabled according to length :
585 265

Pst I sites in INTERFERON

CTGCAG (c5)
266

Resulting fragment sizes :
270 580
Tabled according to length :
580 270

Rsa I sites in INTERFERON

GTAC (c2)
533 717

Resulting fragment sizes :
539 179 132
Tabled according to length :
539 179 132

Sma I sites in INTERFERON

CCCGGG (c3)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Sfa NI sites in INTERFERON

GATGC (cX0)
310 639

GCATC (cX0)
0

Resulting fragment sizes :
309 379 162
Tabled according to length :
379 309 162

Sac I sites in INTERFERON

GAGCTC (c5)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Sac II sites in INTERFERON

CCGCGG (c4)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Sac III sites in INTERFERON

ACGT (cX0)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Sal I sites in INTERFERON

GTCGAC (c1)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Sph I sites in INTERFERON

GCATGC (c5)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Taq I sites in INTERFERON

TCCGA (cl)
9

Resulting fragment sizes :
9 841
Tabled according to length :
841 9

Xba I sites in INTERFERON

TCTAGA (cl)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Xho I sites in INTERFERON

CFCGAG (cl)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Xho II sites in INTERFERON

AGATCC (cl)
0
AGATCT (cl)
629
GCATCC (cl)
0
GGATCT (cl)
0

Resulting fragment sizes :
629 221
Tabled according to length :
629 221

Xma I sites in INTERFERON

CCCGGG (cl)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Xma III sites in INTERFERON

CGGCCC (cl)
0

Resulting fragment sizes :
850
Tabled according to length :
850

Bgl I sites in INTERFERON

GCCNNRNNGGC (c7)
0

TthIII I sites in INTERFERON

GACNNNGTC (c4)
0

Ecc B sites in INTERFERON

~~TCANNNNNNNNGTC~~ (cx0)
0

TCANNNNNNNNTGCT (cx0)
0

ASCANNNNNNNNTCA (cx0)
0

Ecc K sites in INTERFERON

AACNNNNNNNGTGC (cx0)
0

GCACNNNNNNNTT (cx0)
0

INTERFERON


MetThrAsnLysCysLeuLeuGlnIleAlaLeuLeu
 GCAACCUUUCGAGCCUUUGCCUCUGGCACACACAGGUAGUAGCGGACACUUGUCUUGUCUACACAGUCUCCUCCAAAUUGCUCUCUCCUG 101
 LeuCysPheSerThrAlaLeuSerMetPheTyrAsnLeuLeuGlyPheLeuGlnArgSerSerAsnPheGlnCysGlnLysLeuLeuTrpGlnLeuA
 UUGUGCUUUCUCCACUACAGCUCUCCUUGCCAUAGGACUACAACUUGCUUGGAUUCUACAAAGAGCAGCAAUUUUACAGUCUACAGCCUUGGSCAAUUGA 201
 snGlyArgLeuGluTyrCysLeuLysAspArgMetAsnPheAspThrProGluIleLysGlnLeuGlnGlnPheGlnLysGlnAspAlaLeuLeuTh
 AUGGGAGGCUUGAUACUGCCUCAAGGACAGGAUGAACUUUGACACCCCUAGGAGAUUAGCAGCUGCAGCAGUUCACAGAGGAGGAGCUGCCGCAUUGAG 301
 rIleTyrGluMetLeuGlnAsnIlePheAlaIlePheArgGlnAspSerSerSerThrGlyTrpAsnGluThrIleValGlnAsnLeuLeuAlaAsnVal
 CAUCUAUGAGAGUCCACAGAACAUUUUGCUAUUUUUCAGACAGAUUCAUUCUACGACUGCCUGGAAUUGAGACAUUUGUUGAGAGAACCCUGGCUAAUGUC 401
 TyrHisGlnIleAsnHisLeuLysThrValLeuGluGluLysLeuGluLysGlnAspPheThrArgGlyLysLeuMetSerSerLeuHisLeuLysArg
 UAUCAUCAGAUAAACCAUCUGACAGACAGUCCUGGACAGAAACUUGGAGAAAGAAAGAUUUUACCCAGCGGAAACACUCAGAGGACGACGUCUGCAAGAGAU 501
 yrTyrGlyArgIleLeuHisTyrLeuLysAlaLysGlnTyrSerHisCysAlaTrpThrIleValArgValLysIleLeuArgAsnPheTyrPheIleAs
 AUUAUGGGAGGAUUCUGCAUUAUCCUGAACGCCACAGGAGUACAGUCACUGGACCCUUGGACCAUAGUCAGAGUAAAUCCUAGGAAACUUUUACUUCAUUA 601
 naArgLeuThrGlyTyrLeuArgAsn
 CAGACUUUACAGGUUACCUCCGAAACUUGAAGAUUCUCCUAGCCUGUGCCUUCUGGACUGGACAAUUGCUUCCAGCUUCCUACACCGACAGUCCUGUUUA 701
 GUGACUGAUGGCUAUGUACUGCAUUGCAAGACACUAGACAUUUUUGAAAUUUUUGAGCUAUUUUUAUUUUUAUUUAUUUAUUUAUUUUUUUUUU 800
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 en présence de l'examineur

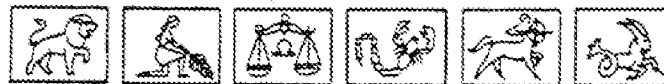
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This is EXHIBIT FIERS-16
 to
 the Affidavit of Walter C. Fiers
 sworn before me
 this 14th day of November, 2001

Commissioner for Oath or Notary Public

DATE FILED: 05/06/2009 02
DOCUMENT NO: 47

OPPOSITION TO EUROPEAN PATENT NO. 0 341 313 OF BIOGEN INC.
SCHERING AKTIENGESELLSCHAFT
APPEAL FILE NO. T0207/94-334
Commissioner of Patents
en présence de l'examinateur

DECLARATION OF DR. MICHAEL HOUGHTON

I, MICHAEL HOUGHTON, declare and state as follows:

1. I am a citizen of the United Kingdom, residing in Danville, California (U.S.A.).
2. I presently hold the position of Director, Non-A Non-B Hepatitis Research at Chiron, Inc., Emeryville, California, where I have been employed since 1982.
3. I obtained a B.Sc. (Honors) in Biological Sciences from the University of East Anglia in 1972. I obtained a Ph.D. in Biochemistry in 1977 from Kings College, University of London.
4. I am an author or co-author of over 150 research papers that have been published in peer reviewed journals. These papers span my work on the interferon gene system, the acetylcholine receptor, the immunoglobulin E gene organization, viral hepatitis C (HCV), and hepatitis delta (HDV). My publications also include work on eukaryote RNA polymerases and transcriptional control. For my contributions to the study of viral hepatitis C, I was a co-recipient of the following awards:

- 1992 Karl Landsteiner Award with Harvey Alter, Daniel Bradley, Qui-Lim Choo, George Kuo and Lacy Overby;
- 1993 Robert Koch Award with Daniel Bradley;
- 1994 William Beaumont Award (American Gastroenterology Association) with Dr. Bradley, George Kuo and Qui-Lim Choo.

A full and comprehensive list of my professional activities, including publications, is set forth in the compilation annexed hereto.

5. From 1972 to 1982 I was employed in the department of Biochemistry and Molecular Genetics of Searle Research and Development, High Wycombe, Buckinghamshire. From 1978 until 1982, I was the project leader of the Human Fibroblast Interferon Genetics project. This project initially involved cDNA cloning, sequencing, and expression of the rare human fibroblast interferon mRNA. Investigations were also performed on the structure of the fibroblast interferon gene within the human chromosome. My group was the first to publish and file a patent application on the partial cDNA sequence and the genomic organization of the fibroblast interferon gene. See, e.g., Houghton et al., Nucl. Acid Res., 8:1913-1931, 1980; Houghton et al., Nucl. Acid Res., 8:2885-2894, 1980; Houghton et al., Nucl. Acid Res., 9:247-266, 1980.

This is EXHIBIT FIERs-30

to

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the Affidavit of Walter C. Fiers
sworn before me
this 13th day of November, 2001

Commissioner for Oath or Notary Public

SUGANO EXHIBIT 1011
FIERS V. SUGANO
INTERFERENCE NO. 105,661

6. Six patent applications were filed by our group in 1980 claiming IFN- β related inventions. The applications were filed on Feb. 6, 1980, Feb. 28, 1980, April 17, 1980, April 24, 1980, May 12, 1980, and Nov. 18, 1980. The first five applications contained IFN- β cDNA sequence information, beginning with a partial sequence in the Feb. 6 application and ending with a completed cDNA sequence disclosed in the May 12, 1980 application. We filed quickly and successively on the cDNA sequencing aspect of the project, in intervals of about three weeks or less. Expression of biologically-active was IFN- β achieved within about two months from determining the complete IFN- β cDNA sequence.

7. I am informed that Biogen is proprietor of European patent 0 041 313 which claims expression of biologically- and immunologically-active interferon in unicellular host cells and that this patent is being opposed by Schering AG. I have read and understood pages 14-21 of Biogen's observations, dated December 21, 1994, in which they contend the skilled worker having the Taniguchi [D2] sequences in hand and attempting to express recombinant IFN- β , would have had serious concerns about the expressibility of the IFN- β DNA sequence in view of its content of hydrophobic amino acids, three cysteine residues (positions 17, 31, and 141), AUA codon for 2 isoleucine residues, an AUG codon at the start of the mature polypeptide, and other similar problems related to protein composition.

8. Prior to June 6, 1980, I knew the complete nucleotide and amino acid sequence of IFN- β , including that it possessed three cysteine residues, hydrophobic amino acids, an N-terminal methionine at the start of the mature protein, and two AUA codons for isoleucine. This specific knowledge of these characteristics of IFN- β 's sequence did not deter me from continuing our efforts to express the cDNA encoding it in *E. coli*. Despite this knowledge, we expected that a significant amount of biologically-active IFN- β would be expressible in bacteria.

9. Once we had obtained the complete IFN- β cDNA, its expression in *E. coli* was routine and straightforward. In fact, expression of biologically-active IFN- β was achieved at our very first attempt, without performing any manipulations to overcome any of the so-called problems, e.g., hydrophobicity, odd number of cysteines, or AUA codons, described in Biogen's observations.

DATE: x May 23rd 1996

x M Houghton

CURRICULUM VITAE

MICHAEL HOUGHTON

Age: 44
Date of Birth: 6th February, 1951
Nationality: UK
Residence: Permanent resident of USA
Marital Status: Married (with two children)

Education

1969-1972 B.Sc. (Honors) Biological Sciences
University of East-Anglia,
Norwich, England

1973-1977 Ph.D. Biochemistry
King's College,
University of London,
England

Posts

1977-1982 Senior Research Investigator - Human interferon genetics
Searle Research Laboratories
Buckinghamshire, England

1982-present Director, Non-A, Non-B Hepatitis Research
Chiron Corporation
4560 Horton Street
Emeryville, California 94608 USA

Honors

Co-recipient of 1991 Karl Landsteiner Award from the American Association of Blood Banks for Hepatitis C Viral Research
Recipient of the Robert Koch Award from Germany
Recipient of the Williams Beaumont Prize from the American Gastroenterology Association
Honoree of the Japanese Medical Congress

Patents

Numerous patents issued in the fields of recombinant human interferons, bacterial expression vectors, Hepatitis C and D viruses.

Publications

Over 150 publications in the fields of gene regulation, human beta interferon and hepatitis C and D viruses.

Publications

Transcriptional and translational control in eukaryotes

1. "The purification and properties of hen oviduct Form B DNA-dependent RNA polymerase" M. Houghton and R.F. Cox (1974) Nucl. Acids Res. 1, 299-308.
2. "The presence of ovalbumin mRNA coding sequences in multiple restriction fragments of chicken DNA" M.T. Doel, M. Houghton, E.A. Cook and N.H. Carey (1977) Nucl. Acids. Res. 4, 3701-3713.
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13. "The complete amino acid sequence of human fibroblast interferon as deduced using synthetic oligodeoxyribonucleotide primers of reverse transcriptase" M. Houghton, M.A.W. Eaton, A.G. Stewart, J.C. Smith, S.M. Doel, G.H. Catlin, H.M. Lewis, T.P. Pate, J.S. Emtage, N.H. Carey and A.G. Porter (1980) Nucl. Acids Res. 8, 2885-2894.
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